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14. ABSTRACT We hypothesize that the biomechanical perturbations of the brain that yield blast-induced mTBI in injured warfighters can be recreated with reasonable fidelity in rats under carefully controlled experimental conditions, and that several of the characteristic sequelae of blast-induced mTBI observed clinically can be reproduced in a rodent injury model. In many, if not most circumstances yielding blast mTBI, brain injury results from a combination of blast overpressure (BOP) (i.e. primary blast) and head acceleration and/or impact (i.e. tertiary blast). The mTBI resulting from these combined insults may be fundamentally different from that seen from either insult alone.					
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INTRODUCTION

Many warfighters who sustain blast-induced TBI in combat are exposed to a brain insult resulting from a combination of both a shock wave and biomechanical perturbation related to rapid acceleration and/or impact with a solid object (MacDonald et al., 2011). The TBI resulting from these combined insults is likely to be fundamentally different from that seen from either insult alone. We hypothesize that the combined biomechanical perturbations of the brain that yield blast-induced mild TBI in injured warfighters can be recreated with reasonable fidelity in rats under carefully controlled experimental conditions, and that several of the characteristic sequelae of blast-induced mild TBI observed clinically can be reproduced in an established rodent injury model. We anticipate that this model can provide a valuable experimental tool to assist ongoing efforts to mitigate the risks and consequences of blast-induced mTBI in warfighters.

OVERALL PROJECT SUMMARY

Research accomplishments associated with each task outlined in the approved Statement of Work are described below.

Task 1

Manipulate and monitor blast exposure conditions (i.e. incident flow conditions) in the compression-driven shock tube and recreate with reasonable fidelity the biomechanical loading conditions estimated to underlie primary blast-induced mild TBI in warfighters. Establish a mild injury severity based upon loss of consciousness (LOC), histopathology, and neurological and neurobehavioral outcomes.

Task 1 progress: Over the course of this project, several modifications in blast exposure conditions were implemented to improve the reproducibility and fidelity of blast simulation in the cylindrical shock tube. In particular, artefactual biomechanical loading associated with end jet effects was eliminated and uncontrolled acceleration and displacement were recognized and have been restricted by tautly suspending experimental subjects in coarse mesh netting. Nevertheless, we learned that cylindrical shock tubes are inherently and universally limited for blast simulation. Notably, in the absence of an end wave eliminator, negative phase and recompression waves are artefacts of the rarefaction from the end of the open tube and the secondary shock is moving in the reverse direction (upstream not downstream). Additionally, without a reflection eliminator, waves reverberate throughout the length of the tube after the passage of the initial shock front (Ritzel et al., 2011). Finally, rather than the sharp peak positive pressures associated with the Friedlander waveform, cylindrical shock tubes typically produce plateau or flat-top waveforms with relatively long durations (6-12 msec, illustrated below in fig 1).

With a divergent transition section and an end wave eliminator, a recently acquired advanced blast simulator (ABS) eliminates these artefacts along with the positional heterogeneity in pressures and flow conditions encountered in cylindrical shock tubes (Ritzel et al., 2011). In the ABS (fig 1), positive pressure durations can be reduced to 1-2 msec, which may better represent waveforms resulting from IEDs. We strongly believe that it is important to perform several key experiments using the new ABS to confirm results, particularly since the flow conditions are quite different between the two devices (fig 2) and the means by which rats were secured in the old shock tube and exposed to blast overpressure evolved substantially over the duration of the project as we became more aware of the impact of set-up variables on experimental measures and outcomes. Confirmation of results obtained with the cylindrical shock tube by blast simulations in the ABS will validate the findings and also provide valuable insights for their interpretation. As noted previously, bad blast simulations have confounded much of the preclinical biomedical blast literature to date, and we strongly desire to correct that situation. An animal use protocol was prepared to directly compare the loading conditions and outcomes resulting from blast overpressure exposures in the cylindrical shock tube and ABS.

Although no additional animal experiments were performed during this reporting period, we did continue analyses of telemetric EEG recordings which have shifted from detection of post-traumatic seizure activity, which proved to be infrequent and variable, to longer-term spectral shifts to monitor for other EEG anomalies over a more prolonged timeframe. In addition, neurobiological and neurochemical analyses were continued using previously collected tissue samples and extracts. In particular, these measurements targeted neuroinflammatory mediators and phosphorylated tau protein, which has been proposed to be a disruptive change linked to chronic traumatic encephalopathy (CTE).

Task 2

Establish conditions yielding a mild injury severity with a surgery-free adaptation of the weight drop brain injury model (or alternative) to create tertiary blast brain injury based upon LOC, histopathology, and neurological and neurobehavioral outcomes.

Task 2 progress: As previously described, the Marmarou weight drop technique was adapted to create tertiary (i.e. impact-acceleration) injury, and the neurobehavioral disruptions and brain histopathological consequences of these weight drop exposures have been thoroughly characterized over a range of drop heights. Although no animal experiments were performed during this reporting period, proteomic and genomic assessments were performed with previously collected tissue samples to provide insights into the neurobiological underpinnings of these brain injuries.

Task 3

Combine blast overpressure (BOP) and the selected impact acceleration insult at multiple combined severities, and evaluate the histopathological, physiological, and neurobehavioral outcomes relative to those seen following each insult alone. Establish combined injury conditions to produce mTBI.

Task 3 progress: Injuries produced by combinations of BOP and weight drops of varied heights were compared to those generated by exposures to closely coupled repeated blasts (separated by 1 min) during the previous reporting period. With either combination, neurobehavioral deficits were recorded that were greater and more persistent than produced by a single insult alone. These findings are consistent with the primary hypothesis of the project, namely that the TBI resulting from these combined insults is fundamentally different from that seen from either insult alone. Using tissue samples and EEG data collected from these experimental subjects, a variety of neurochemical measurements, histopathological evaluations, EEG recordings, and novel imaging comparisons were analyzed to extend the comprehensive characterization of these injuries and shed light on underlying neurobiological mechanisms and the means by which these insults might interact.

Task 4

Using a mach stem wedge equipped with a high velocity piston impactor, instantaneously combine impact acceleration with BOP within the shock tube to produce and evaluate the concomitant combined effects of primary and tertiary blast relative to those seen following each insult alone. Establish a mild injury severity based upon loss of consciousness (LOC), histopathology, and neurological and neurobehavioral outcomes.

Task 4 progress: With acquisition of an advanced blast simulator (ABS), an animal use protocol was written and a rat holder incorporating a pneumatically driven piston was designed to enable near instantaneous blast and impact to be combined for improved fidelity of combined insults.

KEY RESEARCH ACCOMPLISHMENTS

Bulleted list of key research accomplishments emanating from this research.

- An advanced blast simulator (ABS) has been established as a means to generate substantially improved high fidelity blast simulations relative to those achieved with a traditional cylindrical shock tube.
- In preparation for rat exposures, blast flow conditions produced by the ABS have been characterized using pressure recordings and high speed videography of inanimate objects exposed to blast overpressure (including surrogate rats).

- Identified that the putative mediator of chronic traumatic encephalopathy, phosphorylated tau protein (*pTau*), is significantly elevated in different brain regions after either blast exposure or weight drop injury in close association with decreased expression and activity of tissue non-specific alkaline phosphatase (TNAP), the enzyme primarily responsible for dephosphorylating *pTau* and restoring microtubule assembly for regeneration. These findings point to a neurobiological link between acute brain injuries and this tau-protein linked neurodegenerative disorder.
- Identified large and sustained blast-induced elevations of the neuroinflammatory mediator chemokine ligand 2 (CCL2) in brain and CSF in association with increased expression of its receptor. These findings point to another likely neurobiological link between sustained neuroinflammatory processes triggered by acute brain injuries and the pathogenesis of chronic neurodegenerative disorders.
- Algorithms have been developed and further refined to analyze EEG recordings through 30 days post-injury to distinguish electrophysiological consequences of individual and combined blast- and weight drop-induced brain insults.
- An animal use protocol was prepared and submitted for IACUC and ACURO review and approval to directly compare the effects on rats of blast exposures in the ABS with blast exposures in the cylindrical shock tube.

REPORTABLE OUTCOMES

Reportable outcomes that have resulted from this research include the following.

Manuscripts and Book Chapters

Kamnaksh A, Ahmed FA, Kovesdi E, BarryES, Grunberg NE, Long JB, and Agoston DV. Molecular mechanisms of increased cerebral vulnerability after repeated mild blast-induced traumatic brain injury. *Translational Proteomics* 3 (2014) 22-37.

Vogel, EW, Morrison B, Evilsizor MN, Griffiths DR, Thomas TC, Lifshitz, J, Sutton, RL, Long, JB, Ritzel, D, Lings, GSF, Huh, J, Raghupathi, R, McIntosh, TK. Experimental Models of Traumatic Brain Injury: Clinical Relevance and Shortcomings. In: "Cell Therapy for Neurologic Injury", C. Cox (ed). CRC Press, in press.

Arun P., Oguntayo S., VanAlber S., Gist I., Wang Y., Nambiar M., Long J. Tissue non-specific alkaline phosphatase in the etiology of tauopathy and chronic traumatic encephalopathy. Submitted to *Neuroscience*.

Oral Presentations

Peethambaran Arun: *Biological Dosimeters of Blast Exposure*. Presented at the International State-of-the-Science Meeting on the Biomedical Basis for Mild Traumatic Brain Injury (mTBI) Environmental Sensor Threshold Values (4-6 November 2014, McLean VA).

Poster Presentations:

P. Arun, D. Wilder, A. Edwards, Y. Wang, I. Gist, J. B. Long. Blast exposure phosphorylates Tau preferentially at serine396, which can trigger Alzheimer's-like pathology. National Neurotrauma Symposium held at San Francisco, CA- July 2014

P. Arun, D. Wilder, A. Edwards, Y. Wang, I. Gist, J. B. Long. Phosphorylation of Tau After Blast Exposure: A Potential Predisposition to Alzheimer's-like Pathology. Society for Neuroscience annual meeting held at Washington, DC - November 2014.

Y. Wang, Y. Wei, L. Tong, A. Edwards, I. Gist, P. Arun, and J. Long. Characterization of blast-induced auditory and vestibular injury in rats. Society for Neuroscience annual meeting held at Washington, DC - November 2014.

Y. Wang, Y. Wei, L. Tong, P. Arun, A. Edwards, S. Oguntayo, I. Gist and J. Long. Characterization of Blast-Induced Vestibular Injury in Rats. The Annual Symposium for Neurotrauma Society San Francisco, CA June 2014

Y. Wang, Y. Wei, P. Arun, S. Oguntayo, D. Wilder, I. Gist and J. Long. Brain Pathological and Biochemical Responses Following Repeated Blast Exposures in Rats. International State-of-the-Science Meeting on the Biomedical Basis for Mild Traumatic Brain Injury (mTBI) Environmental Sensor Threshold Values (4-6 November 2014, McLean VA).

Research proposals

Based upon work supported by this award, funding was sought through a number of research preproposals and proposals submitted to the CDMRP, DMRP, and MRMC BAA during this reporting period. The research proposal "Assessment and Treatment of Blast-Induced Auditory and Vestibular Injuries" was selected for funding as a Clinical Rehabilitative Medicine Research Program (CRM RP) Neurosensory Research Award within the Defense Health Program/Defense Medical Research Development Program. Co-Is are Dr. Ying Wang and Dr. Peethambaran Arun of WRAIR and Dr. Matthew

Kelley, Dr. Joseph Burns, and Dr. Weise Chang of the National Institute on Deafness and Other Communication Disorders.

CONCLUSION

Results to date are consistent with the hypothesis that BOP generates a closely-associated insult to the brain (and other organs as well) and interactively compromises the brain's resilience and exacerbates the pathophysiological effects of other injury modalities such as impact acceleration (i.e. tertiary injury). With continued refinement, including BOP exposures in the recently acquired ABS, under carefully controlled experimental conditions the combined biomechanical perturbations of the brain that yield blast-induced mild TBI in injured warfighters can be recreated with reasonable fidelity to reproduce characteristic sequelae of blast-induced mild TBI. The endproduct model will provide an invaluable tool to define underlying neurobiological mechanisms and rationally establish effective countermeasures to lessen short-term impairments (e.g. return-to-duty) as well as chronic debilitation (e.g. chronic traumatic encephalopathy).

REFERENCES

- Mac Donald CL, Johnson AM, Cooper D, Nelson EC, Werner NJ, Shimony JS, Snyder AZ, Raichle ME, Witherow JR, Fang R, Flaherty SF, Brody DL. Detection of blast-related traumatic brain injury in U.S. military personnel. *N Engl J Med*. 2011 Jun 2;364(22):2091-100.
- Marmarou A, Foda MA, van den Brink W, Campbell J, Kita H, Demetriadou K. A new model of diffuse brain injury in rats. Part I: Pathophysiology and biomechanics. *J Neurosurg*. 1994 Feb;80(2):291-300.

APPENDICES

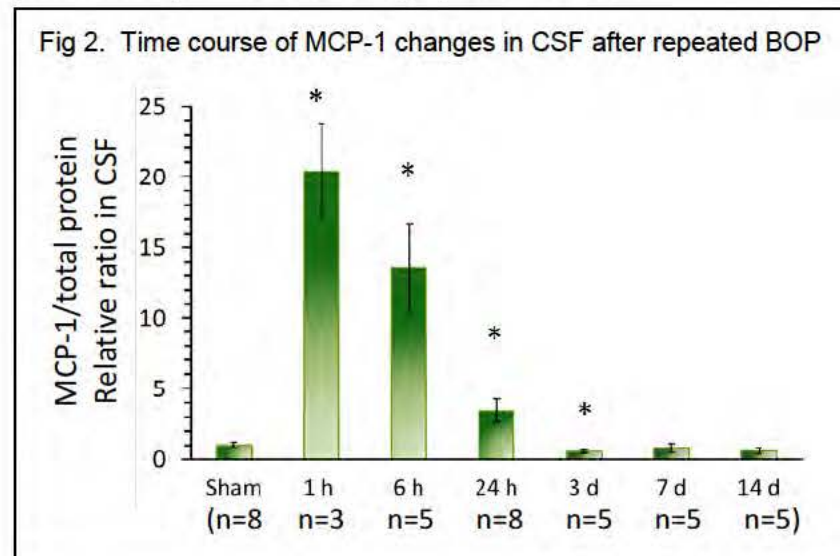
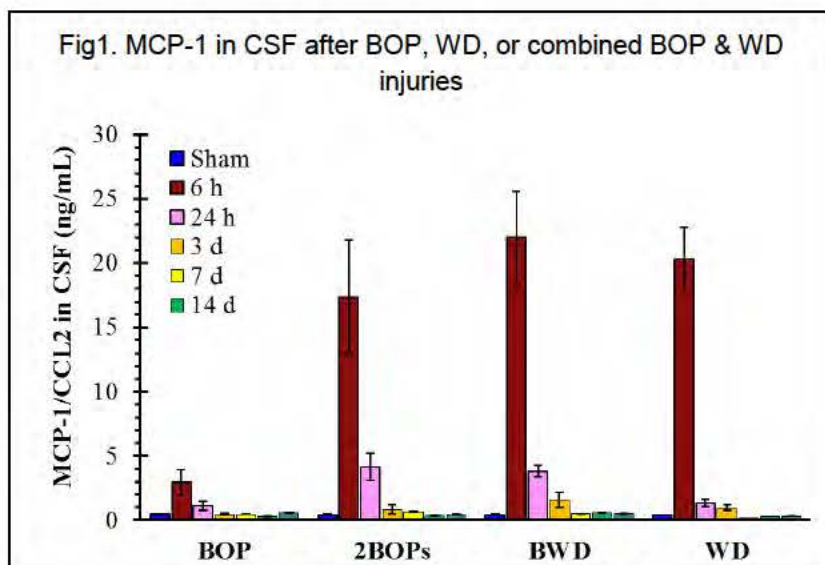
1. Supporting data
2. Published manuscripts

SUPPORTING DATA

We have established several important neurobiological responses to blast and/or weight drop (tasks 1-3) during this reporting period.

Neuropathological changes are temporally and anatomically closely associated with neuroinflammation, prompting interest in the latter as a target for therapies to ameliorate

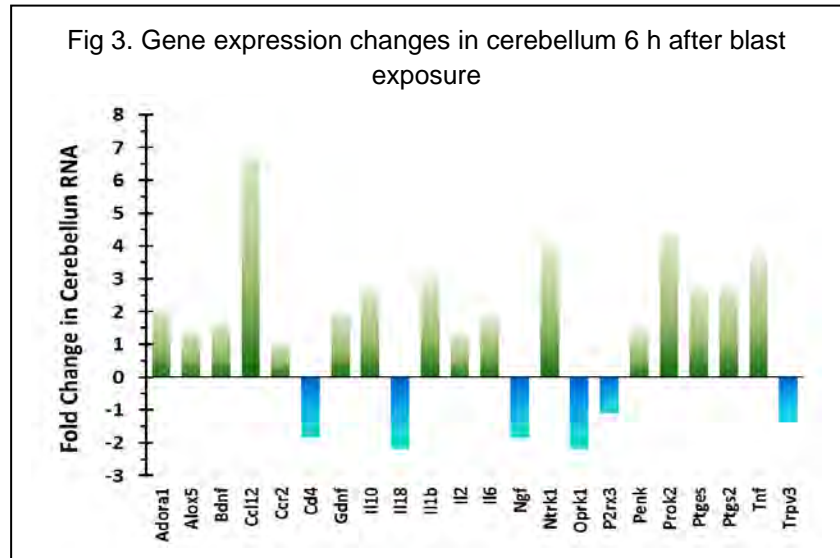
injury mechanisms. It is well known that cytokines including chemokines (CC), interferons, interleukins (IL) and tumor necrosis factor (TNF) play crucial roles in inflammatory development (reviewed in Gyoneva and Ransohoff, 2015). Chemokine ligand 2 (CCL2) exhibits a chemotactic activity for monocytes and basophils. It is primarily secreted by monocytes, macrophages, dendritic cells in peripheral organs and is also expressed by neurons, astrocytes and microglia. CCL2 is also referred to as monocyte chemoattractant protein-1 (MCP-1) and small inducible cytokine A2. MCP-1/CCL2 recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection.



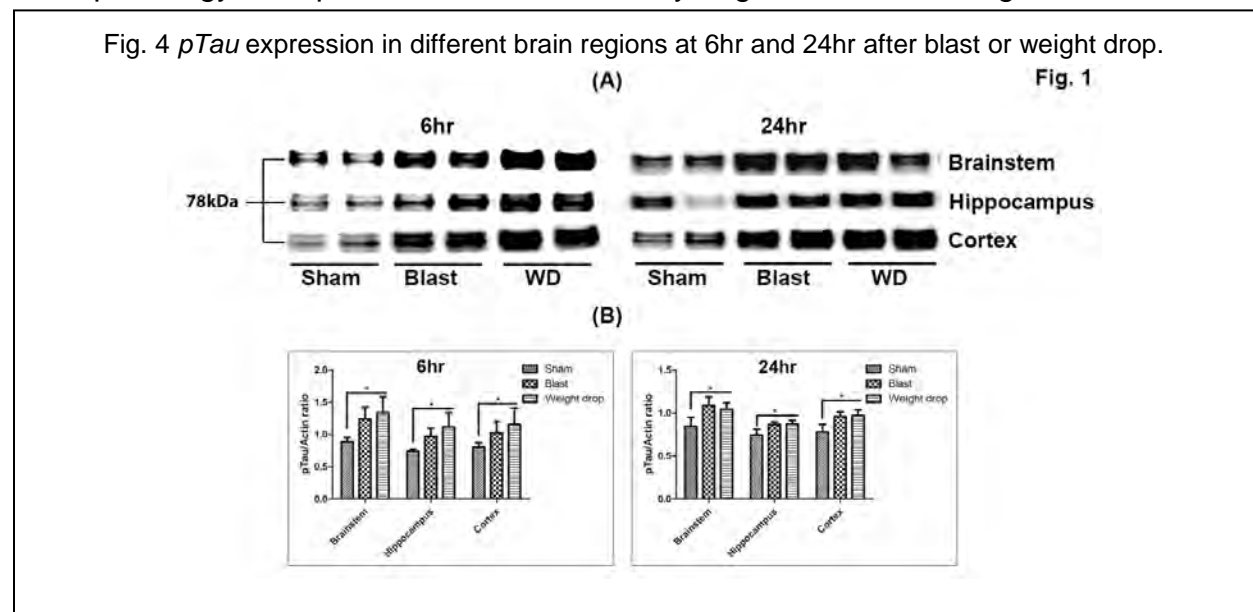
To explore neuroinflammation after BOP exposures, we have measured changes in levels of MCP-1 in CSF, plasma and brain tissue, and we see sizeable increases that are appreciably larger with two blast exposures than with one (fig 1-2). Changes in MCP-1 levels were also much greater in CSF and brain tissues than were

associated changes in plasma at 6 h and 14 h after blast exposures. In addition, blast exposures induced changes in expression of multiple genes in the CNS (fig 3). The

inflammation related genes CCL12, IL10, IL6, IL1b and TNF were up-regulated, while IL8 was down-regulated at 6 h after TBI. Modulation of these mediators and associated inflammatory pathways in the disrupted milieu of the injured brain may ameliorate the maladaptive sustained neuroinflammation driving secondary injury cascades, and thereby promote recovery and long-term improvements.



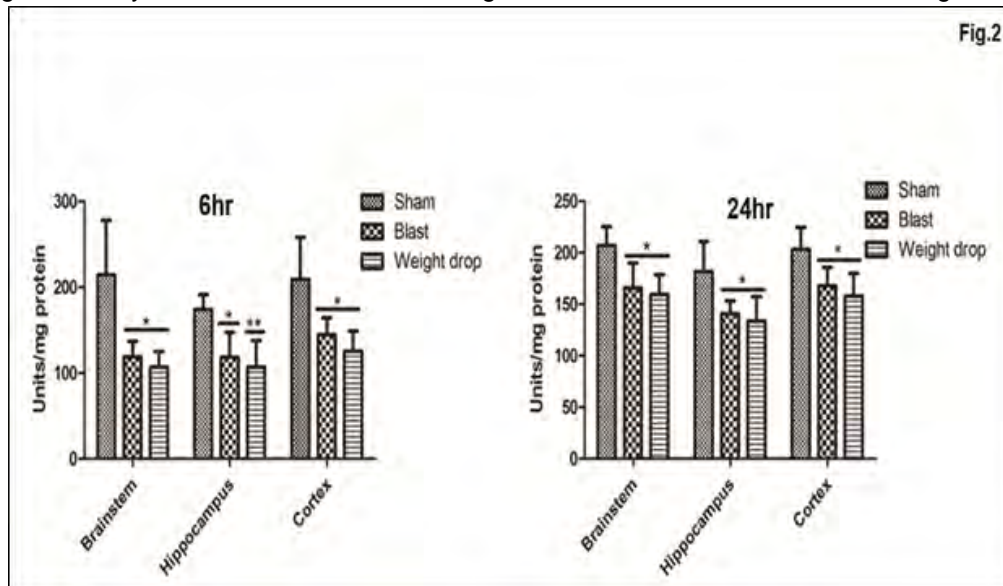
Blast exposure has been linked to chronic traumatic encephalopathy (CTE), a tau protein-linked neurodegenerative disorder which has been observed in several athletes with a history of multiple concussions. In particular, phosphorylated *Tau* (*pTau*) protein neuropathology, with perivascular neurofibrillary degeneration, is recognized as a



distinct feature of CTE and has been observed postmortem in the brains of blast victims and contact-sport athletes. Phosphorylation of *Tau* protein disrupts microtubule assembly in neurons which can result in tauopathy and the formation of neurofibrillary tangles seen in neuro-degenerative disorders such as Alzheimer's disease (AD) (Hanger et al., 1991, Iqbal et al., 1994, Wang et al., 1996). Dephosphorylation of *pTau* is critical to prevent tauopathy and to restore microtubule assembly for

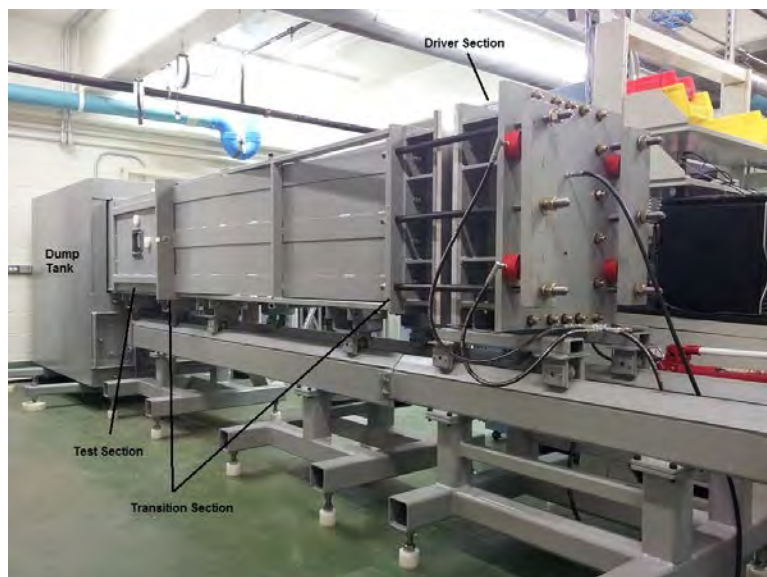
neuroregeneration. Tissue nonspecific alkaline phosphatase (TNAP) is the enzyme principally responsible for dephosphorylating *pTau* in neurons, and in so doing, TNAP may play a major role in the etiology of brain disorders involving this neuropathological feature.

Fig. 5. Activity of TNAP in different brain regions at 6hr and 24hr after blast or weight drop.



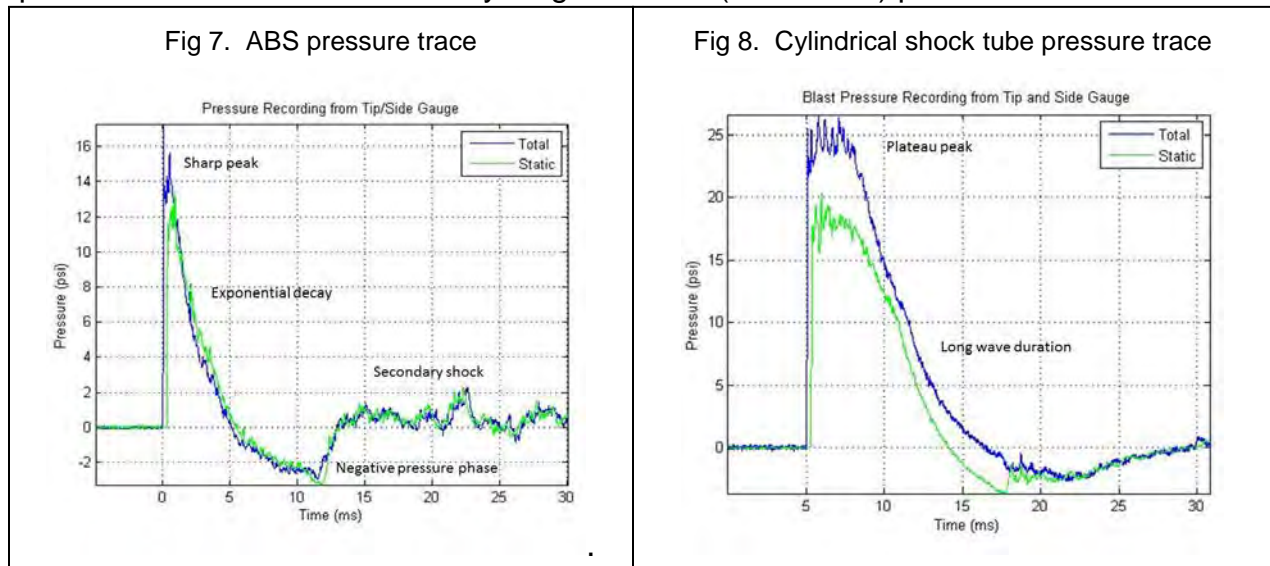
As described in a submitted manuscript under review, brain injury after blast exposure or weight drop resulted in significant increases in the accumulation of *pTau* in different brain regions which were closely associated with decreased expression and activity of the enzyme tissue nonspecific alkaline phosphatase (TNAP). The decreased activity of TNAP in the brain after injury was associated with a similarly significantly decreased total alkaline phosphatase activity in the plasma. In view of the known function of TNAP in dephosphorylating *pTau*, the accumulation of *pTau* after brain injury could be attributed to the decreased TNAP activity/levels in the brain

Fig 6. Advanced blast simulator.



after the injury, and point to the importance of this enzyme in the evolution of neurobiological events resulting in CTE.

We recently acquired an advanced blast simulator (ABS, fig 6), which generates a high fidelity Friedlander waveforms closely resembling that produced by explosives in the open field. Comparison of these pressure tracings highlights the contrast between the “Friedlander-like” sharp peak positive pressures produced in the ABS (fig 7) and the plateau wave-forms with relatively long durations (6-12 msec) produced in this and



other cylindrical shock tubes (fig 8), which will yield unscaled drag forces greatly exceeding those occurring with an explosion in the free field. In addition, the difference between the total and static pressure recordings (blue vs green tracings in figs 7 and 8) is much less in the ABS than in the cylindrical shock tube. This difference between total and static pressure is the dynamic pressure which gives rise to blast wind resulting from the kinetic energy imparted to the air as it is traversed by the shock wave. In addition to creating a higher fidelity shock wave, the ABS is equipped with optical windows and gauge ports which allow high speed video and pressure recordings to capture the flow conditions and response of the test subject (i.e. translation, acceleration, deformation, etc.).

Supporting Data References:

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Molecular mechanisms of increased cerebral vulnerability after repeated mild blast-induced traumatic brain injury

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Abbreviations: AD, amygdala; BBB, blood brain barrier; bTBI, blast-induced TBI; CNS, central nervous system; DHC, dorsal hippocampus; ICV, increased cerebral vulnerability; mTBI, mild traumatic brain injury; OF, open field; PFC, prefrontal cortex; PTSD, post-traumatic stress disorder; rmTBI, repeated mild traumatic brain injury; TBI, traumatic brain injury; USU, Uniformed Services University; VHC, ventral hippocampus.

ABSTRACT

The consequences of a mild traumatic brain injury can be especially severe if it is repeated within the period of increased cerebral vulnerability (ICV) that follows the initial insult. To better understand the molecular mechanisms that contribute to ICV, we exposed rats to mild blast overpressure at a rate of 1 per 30 min (a total of 5 exposures), monitored select physiological parameters, and assessed their behavior. Two days post-injury or sham, we determined changes in protein biomarkers related to various pathologies in behaviorally-relevant brain regions as well as in the plasma. We found that oxygen saturation and heart rate were transiently depressed following mild blast exposure and that injured rats exhibited significantly increased anxiety- and depression-related behaviors. Proteomic analyses of the selected brain regions showed evidence of substantial oxidative stress and vascular changes, altered cell adhesion, inflammation, as well as neuronal and glial cell loss/damage predominantly in the prefrontal cortex; similar pathological changes were detected in the plasma of injured rats. Our findings indicate that repeated mild blast exposure induces complex molecular pathomechanisms that can be detected in the peripheral blood. The validation and subsequent use of these markers can help identify at-risk individuals and the period of ICV.

Keywords: blast; cerebral vulnerability; neurobehavior; protein biomarkers; traumatic brain injury

1. Introduction

Mild traumatic brain injuries (mTBIs) constitute approximately 80% of all traumatic brain injuries (TBIs) [1]. Among civilians, mTBIs (better known as concussions) affect ~1.3 million individuals in the US annually mostly during contact sports such as boxing, hockey, and football [1, 2]. In the military, ~266,000 service members suffered mTBIs between the years 2000-2012 with the overwhelming majority of injuries being blast-induced (<http://www.dvbic.org/dod-worldwide-numbers-tbi>). Because mTBIs typically result in transient and very mild symptoms, many injured individuals return to the same activity—sports and/or military duty—and significant numbers are re-injured, some multiple times causing repeated mild TBI (rmTBI). While a single mTBI can result in neurobehavioral problems such as increased anxiety and cognitive impairment, the probability of short- as well as long-term impairment is significantly higher following rmTBI [3].

Clinical and experimental data signify that the interval between insults is a key factor in determining outcome severity after rmTBIs [4]. Clinical observations indicate that if re-injury occurs within a specific timeframe of the initial insult, the consequences are significantly more severe and can even be fatal (i.e., second impact syndrome) [5]. Moreover, existing experimental data suggest an inverse relationship between outcome severity and time elapsed between injuries. Two key studies using different models of rmTBI have shown that the initial insult triggers pathological changes such as decreased cerebral glucose metabolism, and increased axonal and vascular vulnerability [6-8]. These otherwise transient changes predispose the brain for additional damage if subsequent insults take place within a specific period of increased cerebral vulnerability (ICV) (days to weeks in humans and hours in rats).

The exact pathobiology of ICV is currently unknown, and in the absence of such knowledge we are unable to identify the “window period” of ICV in order to prevent cumulative brain damage [5, 9]. Our previous works using the rodent model of blast-induced TBI (bTBI) identified some of the functional deficits sustained following single and repeated mild blast overpressure exposure as well as several of the molecular and cellular changes associated with each type of injury [10]. We found that when rats were exposed to mild blast overpressure (~ 138 kPa) once per day for five consecutive days, the resultant damage (as indicated by specific protein biomarker levels in the plasma and in brain tissue) was only moderately greater in multiple blast-injured rats compared to single-injured rats at 2 hours post-injury, 22 days, or even 42 days after the injury. Consistent with the observations from the abovementioned studies, our data indicated that the period of ICV after blast-induced mTBI is considerably shorter than 24 h in rats.

To better understand the biology of ICV, in this study we exposed rats to a total of 5 mild blasts at the rate of 1 per 30 min. As described herein, this increased frequency/shortened interval of insults resulted in significant alterations in physiological parameters, neurobehavioral deficits, and extensive molecular changes that can be detected in functionally-relevant brain regions as well as in circulating blood. The detected changes in protein biomarker levels implicate oxidative stress, vascular pathologies, and inflammation in the cerebral vulnerability that follows blast-induced rmTBI. These markers can be tested in humans who have suffered a mTBI and if validated they will aid in identifying individuals with ICV and in determining a “safe return to duty/play”.

2. Materials and methods

2.1. *Animals*

A total of twenty-two male Sprague Dawley rats (weight at arrival: 280-300 g; approximate age in days: 63-67) (Charles River Laboratories, Wilmington, MA) were used in our study. Upon arrival at the Uniformed Services University (USU; Bethesda, MD), animals were housed in pairs in standard rat cages in a reverse 12 h-light 12 h-dark cycle with food and water ad lib. During the 5 day acclimation period, animals were handled for 5 min each day prior to undergoing baseline physiological monitoring and behavioral testing. Baseline horizontal activity results were used to create the experimental groups: naïve ($n = 4$), sham ($n = 6$), and injured ($n = 12$) with no statistical significance among them. Animals were handled according to protocol, approved by the Institutional Animal Care and Use Committee at USU.

2.2. *Experimental manipulations and injury*

For the duration of the study, naïve animals were kept in the animal facility at USU without any manipulation except on physiological monitoring and behavioral testing days as described later. On the day of the injury, animals in the sham and injured groups were transported from USU to Walter Reed Army Institute of Research (Silver Spring, MD). Sham rats were anesthetized for 6 min in an induction chamber with 4% isoflurane (Forane; Baxter Healthcare Corporation, Deerfield, IL) and placed into the shock tube holder in a transverse prone position without being exposed to blast overpressure. Sham rats were kept in the procedure room adjacent to the shock tube for the length of the injured animals' exposures [10].

Injured animals underwent the same procedures as their respective sham group in addition to being exposed to 5 mild blasts of varying pressures at a rate of 1 per 30 min (*blast no. 1*: Mylar membrane gauge thickness = 750 [average peak total pressure: 15.54 psi or ~107.14 kPa, total

overpressure duration: 9.01 ms]; *blast no. 2*: Mylar membrane gauge thickness = 1400 [average peak total pressure: 19.41 psi or ~133.83 kPa, total overpressure duration: 10.60 ms]; *blast no. 3*: Mylar membrane gauge thickness = 1000 [average peak total pressure: 17.78 psi or ~122.59 kPa, total overpressure duration: 9.22 ms]; *blast no. 4*: Mylar membrane gauge thickness = 750; *blast no. 5*: Mylar membrane gauge thickness = 1000. Blast injury was administered to rats (weight at injury: 320-360 g) while wearing chest protection using a compressed air-driven shock tube as described earlier in detail [10, 11]. Mortality was approximately 8% (i.e., 1 rat). All animals were transported back to the USU animal facility at the conclusion of the exposures.

2.3. *Physiological parameters*

Physiological parameters were measured non-invasively under isoflurane anesthesia at baseline, immediately following injury (x5 for injured rats), and 24 h after blast (or sham) exposure using the MouseOx® Pulse Oximeter adopted for rats (Starr Life Sciences Corp., Oakmont, PA, USA) [12]. The selected parameters included: **a)** arterial oxygen saturation, a ratio of oxyhemoglobin to total hemoglobin concentration in arterial blood (normal O₂ levels: 95-100%; low O₂ levels: < 95%); **b)** heart rate, the number of heartbeats per min (reference range for Sprague Dawley rats: 250-450 beats per min); **c)** pulse distention: a measure of local blood flow; **d)** breath rate, the number of breaths taken per min (reference range for Sprague Dawley rats: 70-115 breaths per min).

2.4. *Behavioral tests: Open Field*

All animals were tested at baseline and 24 h post-injury (or sham) using the open field (OF) system, which measures naturally occurring behaviors that are exhibited when an animal explores and interacts with its surroundings [13]. Variables extracted from the animal's movement within the chambers (i.e., horizontal movement, vertical movement, and time spent in the center of the chamber) provide information about gross motor performance, depression-related behavior, and anxiety-related behavior.

OF activity was measured using Accuscan Superflex Sensor Version 2.2 infrared photocell system in the Accuscan Instruments Standard Animal Cage (measuring 40 x 40 x 30 cm; Accuscan Instruments Incorporated, Columbus, OH) located in a dedicated room designed to minimize acoustic interruptions. The animal's locomotion is captured by three, paired 16-photocell Superflex Sensors that transmit the location data to the Accuscan Superflex Node located on the upper-rear of the chamber. The Superflex Node transmits the OF data from each of the 16 chambers to a computer located within the test room where the data is processed and aggregated by Accuscan Fusion

Software (Version 3.4). The OF activity of each rat was measured for 1 h during its active period (dark cycle).

2.5. *Blood and tissue collection*

Animals were terminated 1 day following the completion of the behavioral testing (i.e., 48 h post-injury or sham). For protein measures, rats (N = 14; naïve = 4, sham = 4, injured = 6) were deeply anesthetized with isoflurane inhalant until a tail or toe pinch produced no reflex movement. Blood was obtained by cardiac puncture, and samples were promptly centrifuged at 10,000 revolutions per min for 15 min at 4°C. The supernatants (plasma) were aliquoted, flash-frozen, and stored at -80°C until processing. Animals were then decapitated using a guillotine (Harvard Apparatus Co.; Dover, MA) and the brains were immediately removed. The prefrontal cortex (PFC), amygdala (AD), ventral hippocampus (VHC), and dorsal hippocampus (DHC) were dissected over wet ice and the dissected brain regions were then flash-frozen and stored at -80°C until processing [10, 14].

2.6. *Protein measures*

Relative concentrations of the selected protein biomarkers (Supplementary Table) were determined using reverse phase protein microarray. Sample preparation, printing, scanning, and data analysis were performed as described in detail [14-17]. Briefly, plasma samples were diluted 1:10 then subjected to an 11-point serial 1:2 dilution and transferred into 384 well plates (Genetix, X7022). Dissected brain regions were pulverized in liquid nitrogen and then sonicated in the presence of protease and phosphatase inhibitors. Protein concentrations were measured using a bicinchoninic acid assay (Thermo Scientific, PI-23250). Samples were diluted in print buffer to a final protein concentration of 1 mg/mL and printed on ONCYTE® *Nova* (blood samples) or *Avid* (tissue samples) single-pad nitrocellulose coated glass slides using an Aushon 2470 Arrayer (Aushon Biosystems, Billerica, MA).

Primary antibodies were diluted to 10x the optimal Western analysis concentration in antibody incubation buffer as described [14-16], and used in the dilutions listed (Supplementary Table). Slides were incubated with the primary antibody solutions overnight at 4°C, then washed and incubated with the secondary antibodies Alexa Fluor® 633 donkey anti-sheep (A-21100), Alexa Fluor® 635 goat anti-mouse (A-31574), Alexa Fluor® 647 goat anti-rabbit (A-21245) or rabbit anti-goat IgG (A-21446) from Invitrogen at a 1:6000 dilution in antibody incubation buffer for 1 h at room temperature. Fluorescent signals were measured in a Scan Array Express HT microarray scanner (Perkin Elmer, Waltham, MA), and data were imported into a Microsoft Excel-based bioinformatics program developed in house for analysis [11, 14, 16, 18]. The linear regression of the

log-log data was calculated after the removal of flagged data, which include signal to noise ratios of less than 2, spot intensities in the saturation or noise range, or high variability between duplicate spots (> 10-15%). The total amount of antigen is determined by the Y-axis intercept or Y-cept [18].

2.7. Data comparison and statistical analyses

All animals (N = 21; naïve = 4, sham = 6, injured = 11) were used for the physiological and the behavioral analyses. Paired *t*-tests were used to assess changes in arterial oxygen saturation, heart rate, pulse distention, and breath rate within each group (baseline vs. 24 h); all post-injury values were compared to baseline values in injured rats. To determine differences (if any) in the measured physiological parameters among the three groups, a one-way ANOVA followed by Tukey's HSD test was conducted at baseline and at 24 h. ANOVA (covarying for baseline) followed by Tukey's HSD test were conducted for each of the behavioral variables. OF activity scores were separated into three subscales: horizontal activity, vertical activity, and center time. Comparisons were performed for *Condition* and *Time*, and statistically significant differences were reported where applicable.

A total of 14 animals (naïve = 4, sham = 4, injured = 6) was used for the protein analyses. Differences in the mean protein biomarker levels measured in plasma and in brain tissue were analyzed with ANOVA followed by Tukey's HSD test. Statistical significance was reported for naïve vs. injured and sham vs. injured. For protein levels in the plasma, statistically significant differences are indicated in boldface. For protein levels in the brain, a two sided *p* value of < 0.05 is depicted by one special character, *p* < 0.01 by two, and *p* < 0.001 by three. All statistical analyses were performed using IBM SPSS Statistics 20 software. Tests were two tailed using $\alpha = 0.05$; data are presented as the mean \pm S.E.M.

3. Results

3.1. Oxygen saturation and heart rate are transiently depressed following repeated mild blast sustained at short intervals

A pulse oximeter was used to measure arterial O₂ saturation, heart rate, pulse distension, and breath rate at baseline, after each blast exposure for injured rats, and 24 h post-injury (or sham). Of the four physiological parameters, only arterial O₂ saturation levels and heart rate changed considerably in response to the injuries (Fig. 1A-D). Both arterial O₂ saturation and heart rate significantly decreased in injured rats compared to baseline values. However, this depression was transient as it was followed by a full recovery at 24 h (Fig. 1A-B). No significant injury-induced changes were

measured in pulse distension after any of the exposures while changes in breath rate were minimal and similarly transient (Fig. 1C-D).

3.2. Repeated mild blast overpressure exposure adversely affects motor activity and functional outcome

Using an open field system we determined the effect of repeated mild blast exposure on gross motor activity as well as anxiety- and depression-related behavior. Fig. 2A summarizes the amount of horizontal activity, an index of general health and movement, of animals at baseline and 24 h following injury (or sham). Twenty-four hours post-injury, sham animals (8504.16 ± 901.74) were not significantly different from naïve animals (10661.92 ± 1103.70), but there was a significant effect of injury such that injured animals (6777.67 ± 665.50) had significantly less horizontal activity than naïve animals.

Fig. 2B shows the amount of vertical activity, an index of depression-related behaviors, where less vertical activity indicates more depression-related behaviors. At 24 h post-injury, sham animals (1288.55 ± 209.48) were not significantly different from naïve animals (1862.90 ± 256.25). However, injured animals (887.01 ± 154.38) had significantly less vertical activity than naïve animals. Injured animals (70.86 ± 22.44) also spent significantly less time in the center of the chamber, an index of anxiety-related behaviors (more time in the center indicates less anxiety-related behaviors), than naïve animals (236.73 ± 36.37) (Fig. 2C). Naïve animals were not significantly different from sham animals (140.63 ± 30.12).

3.3. The exposure to mild blasts at short intervals induces complex changes in protein biomarker levels in functionally-relevant brain regions

Two days following injury (or sham), we dissected the PFC, AD, DHC, and VHC and determined the effect of repeated mild blast exposure on tissue levels of select protein biomarkers related to oxidative stress and vascular functions, cellular adhesion and the extracellular matrix, inflammation, and glial cell response.

Of the 2 biomarkers related to oxidative stress, 4-hydroxy-2-noneal (HNE) and hypoxia-inducible factor 1 α (HIF1 α), HNE was significantly increased in all four of the tested brain regions whereas changes in HIF1 α tissue levels were limited to the AD and the DHC of injured rats (Fig. 3A-B). Similar to HNE, vascular endothelial growth factor (VEGF) and aquaporin 4 (AQP4) levels

were significantly increased in all brain regions of injured rats. On the other hand, tissue levels of von Willebrand factor (vWF) were most significantly changed in the PFC only (Fig. 3C-E).

We also detected significant injury-induced changes in the tissue levels of cellular adhesion and extracellular matrix molecules. While integrin $\alpha 6$ tissue levels were mainly elevated in the PFC and the AD of injured rats, TIMP metalloproteinase inhibitor 1 (TIMP1) concentrations were significantly elevated in all brain regions but the DHC (Fig. 4A-B). Of the two extracellular matrix proteins, only TIMP metalloproteinase inhibitor 4 (TIMP4) was significantly increased in all of the tested brain regions compared to naïve and/or sham animals (Fig. 4C).

Among the tested inflammatory biomarkers, only galectin-1 (Gal-1) and macrophage inflammatory protein 1 α (MIP1 α) were significantly higher in all four brain regions of exposed rats (Fig. 4E and Fig. 5C, respectively). Matrix metalloproteinase 8 (MMP8) and chemokine (C-C motif) receptor 5 (CCR5) levels were significantly elevated in all brain regions but the AD (Fig. 4D and 5D, respectively) while toll-like receptor 9 (TLR9), p38 mitogen-activated protein kinase (p38 MAPK), and osteopontin (OPN) tissue levels were elevated in all brain regions but the DHC (Fig. 4F and Fig. 5A-B, respectively).

Both microglia- and astroglia-specific markers (CD53 (OX44) and glial fibrillary acidic protein (GFAP), respectively) showed significant injury-induced increases in injured rats compared to naïve and/or sham animals. OX44 levels were increased modestly in all brain regions but the AD (Fig. 5E). By contrast, GFAP tissue levels increased in all of the analyzed brain regions with the change being among the most robust of the tested biomarkers (Fig. 5F).

3.4. *Some of the molecular changes induced by repeated, high frequency mild blast exposure are reflected in altered plasma levels of protein biomarkers*

Plasma levels of the three tested oxidative stress-related markers, HNE, HIF1 α , and ceruloplasmin, and several vascular protein biomarkers—VEGF, vWF, aquaporin 1 (AQP1), AQP4, fetal liver kinase 1 (FLK1/VEGF receptor 2), and claudin 5—were significantly increased in injured rats compared to naïve, sham, or both control groups (Table 1). Plasma concentrations of cell adhesion and extracellular matrix proteins, integrin $\alpha 6$, TIMP1, and TIMP4, were also significantly elevated in injured animals (Table 2).

Inflammatory markers Gal-1, p38 MAPK, MIP1 α , CCR5, monocyte chemotactic protein 1 (MCP1), cytokine-induced neutrophil chemoattractant 1 α (CINC1 α), fibrinogen, C-reactive protein (CRP), and N-formyl peptide receptor (FPR) were significantly increased in the plasma of injured rats compared to naïve and/or sham animals (Table 3). Consistent with the abovementioned

biomarkers, plasma concentrations of glial—GFAP, OX44, and S100 calcium binding protein 3 (S1003)—and neuronal protein markers—neuron-specific enolase (NSE), neurofilament-heavy chain (NF-H), creatine kinase-brain type (CK-BB), and tau—were all significantly elevated in injured animals two days after blast overpressure exposure (Table 4).

4. Discussion

In this work we have shown that mild blast overpressure repeated at the rate of 1 per 30 min causes significant physiological, neurobehavioral, and molecular changes using our well-established rat model of bTBI. Our findings suggest that oxidative stress, vascular pathologies, and inflammation are critical components of the molecular pathology underlying ICV in blast-induced rmTBI. The biomarkers used in this study are candidates for clinical testing and if verified, they can be developed into blood-based diagnostics to aid in the identification of individuals with ICV.

We selected to monitor changes in physiological parameters using non-invasive methods similar to those utilized in clinical settings. Injury-induced changes in heart and respiratory rates, O₂ arterial saturation, and pulse distention are used as indicators of potential intracerebral pathologies such as disturbed cerebral blood flow, elevated intracranial pressure, and ischemia especially in severe and moderate TBI. These transient post-injury changes, particularly post-traumatic hypoxia, can contribute to ICV. We found that while heart rate remained within the normal range for rats, it was significantly decreased after mild blast overpressure exposure compared to baseline. Similarly, arterial O₂ saturation was significantly lower after exposures than pre-injury values. The mild and transient hypoxia we measured is consistent with previous observations identifying metabolic depression as one of the underlying causes of ICV. Thus, it may be useful to monitor vital signs as they can aid in the detection of altered physiology and the degree of ICV.

Behavioral assessments have shown that mood changes and increased anxiety are two of the major symptoms following bTBI [19, 20]. We previously observed such changes when we exposed rats to a single or multiple mild blasts 24 h apart and found that anxiety- and depression-related changes manifest shortly after injury and are transient in nature [10]. Although at that “low” blast frequency we failed to detect a cumulative effect of repeated blast (and consequently more severe behavioral symptoms) in multiple-injured rats relative to single-injured rats. In our current study, we also found significantly increased anxiety- and depression-related behaviors in injured animals but no conclusions can be made about the role of ICV in the cumulative effect of repeated mild blast exposure due to the absence of a 24 h interval blast group. The only other study assessing the effects of “tightly coupled” mild blast exposures using the rotarod test showed a moderate, albeit transient

cumulative effect of multiple exposures where animals gradually recovered starting at day 5 post-injury [21]. Unfortunately, no behavioral assessments were performed in the two key studies that directly address the involvement of metabolic depression and axonal and vascular changes in ICV [6, 8], although a previous work by the same group has shown increased cognitive deficits after repeated TBI [7].

Clinical observations have indicated that the behavioral symptoms of mTBIs partly overlap with those of post-traumatic stress disorder (PTSD) [19]. However, available experimental data indicate that the molecular pathologies of PTSD and bTBI may be distinct [14, 22, 23]. The observed increase in anxiety indicates that the prefrontal cortex and/or its functionally related structures (the amygdala and the hippocampus) are adversely affected by repeated injury. Experimental research and clinical imaging studies have shown that the prefrontal cortex is involved in mediating symptoms associated with PTSD as well as TBI, and that the PFC is especially susceptible to noxious insults including trauma [24-27]. Accordingly, our proteomics analyses of the PFC, AD, DHC and VHC showed that of the 17 protein biomarkers measured in brain tissue, the expression of 16 changed significantly in the PFC of injured rats. The second most affected brain region was the VHC in which 14 of the 17 markers changed significantly in response to repeated injury followed by the AD (13 markers) and the DHC (11 markers).

The detected changes in tissue levels of the measured markers suggest complex pathological processes as a result of repeated blast overpressure exposure. These include oxidative stress, which can be related to and/or triggered by the relative hypoxia as discussed above. HNE, a byproduct of lipid peroxidation [28, 29], was significantly elevated in all four brain regions of injured rats. Interestingly, HIF1 α was the only marker with unchanged tissue levels in the PFC in response to repeated injury although its levels were significantly elevated in the AD and in the DHC. HIF1 α , a transcription factor, is part of the adaptive and restorative cellular mechanisms that follow various noxious neuronal insults involving hypoxia (e.g., TBI and stroke) [30]. A potential explanation for why HIF1 α levels remained unchanged in the PFC is the relatively early activation of HIF1 α in response to hypoxia, which is within hours and thus outside of our current termination time point (2 days).

Tissue levels of the 3 markers associated with various aspects of vascular function were significantly elevated in the PFC of injured animals. These findings are consistent with previous observations implicating altered vascular function in the period of ICV. Using impact acceleration injury, Fujita et al. (2012) demonstrated that the initial mild insult can result in ICV via vascular hypersensitivity [6]. When the injury was repeated within 3 h of the initial insult, the second mild injury triggered vascular dysfunction as measured by the hypersensitivity of brain vasculature to

acetylcholine stimulation. No such hypersensitivity was detected when re-injury took place 10 h after the initial injury.

In our study, we found that the expression of VEGF, a mediator of multiple endothelial functions including vascular permeability and endothelial proliferation [31, 32], increased significantly in all tested brain regions. Similar to VEGF, AQP4 was elevated in all 4 brain regions. Increased tissue levels of AQP4, a main water channel of the central nervous system (CNS) [33, 34], indicate altered blood brain barrier (BBB) function. Brain edema has been observed after various brain insults including subarachnoid hemorrhage and TBI. The significance of altered BBB permeability following blast-induced rmTBI is currently not known, but increased BBB permeability has been demonstrated after repeated mild athletic TBI. Of the 3 vascular markers, vWF tissue levels were only significantly elevated in the PFC. In addition to its major role in regulating blood coagulation, vWF has additional functions in the inflammatory response to vascular damage [35].

Neuroinflammation, a hallmark of TBI, can be triggered by multiple upstream pathological changes such as vascular damage and altered cellular adhesion [36, 37]. Abnormal cell surface interactions and the activation of various extracellular matrix proteins have been found after CNS injury [38]. The involvement of the extracellular matrix in the pathobiology of blast-induced rmTBI is indicated by the brain region specific increase in integrin $\alpha 6$. As a member of the transmembrane receptor family, integrin $\alpha 6$ plays a vital role in cell adhesion and cell-cell interactions as well as signal transduction from the extracellular milieu into the cell [39]. The role of the extracellular matrix in ICV is further demonstrated by the substantial increase in brain tissue levels of TIMP1 and TIMP4, inhibitors of matrix metalloproteinases [40, 41]. The expression of TIMP1 and TIMP4 in the PFC as well as the AD (and to a lesser extent in the hippocampal sub-regions) is induced at the transcriptional level by several cytokines linking cell surface remodeling to inflammation.

The selected markers perform various functions in the inflammatory process. Consistent with altered TIMP1 and TIMP4 expression, we found elevated tissue levels of MMP8, a matrix metalloproteinase involved in tissue remodeling after injury and chemokine/cytokine activation and inactivation [42]. Gal-1, a β -galactoside-binding protein with intra- and extracellular functions, is heavily involved in the initiation, propagation, and overall mediation of inflammatory processes [43]. Gal-1 overexpression in the PFC of injured animals is indicative of pathological progression in this vulnerable region. Another molecule with a role in the initiation of the neuroinflammatory process is TLR9, a member of the toll-like receptor family [44, 45]. Cytokine signaling by the TLRs and other proteins activates p38 MAPK in the injured tissues. Both TLR9 and p38 MAPK were similarly elevated in the PFC, the AD, and the VHC.

OPN plays a number of roles in immune function that include cytokine production, cell attachment, and cell activation [46]. Another molecule involved in the synthesis and the delivery of pro-inflammatory cytokines is MIP1 α , a member of the Macrophage Inflammatory Protein family of pro-inflammatory chemokines [36, 37, 47-50]. MIP1 α mediates immune response by activating inflammatory cells which then release additional pro-inflammatory cytokines such as IL-1, IL-6, TNF- α [51, 52]. Chronically elevated MIP1 α levels are found in immune disorders of the CNS such as multiple sclerosis. In the injured CNS, MIP1 α and OPN are likely produced by microglia, the resident immune cell of the brain [53, 54]. CCR5 is a receptor for various chemokines including MIP1 α , MIP1 β , and RANTES [55]. Changes in brain tissue levels of CCR5 in response to repeated blast injury were similar to those of MMP8 as it was significantly elevated in the PFC and the two hippocampal sub-compartments but not in the AD. CCR5, a G-protein coupled receptor, is also expressed by macrophages as well as other cells with signaling functions between the nervous and immune systems [51, 56].

CD53 is a marker of microglia and macrophages [50]. While it is possible that there was a macrophage response at this level of repeated injury, we cannot confirm this in the absence of a detailed immunohistochemical analysis. Nonetheless, we detected significantly increased CD53 (OX44) levels in all brain regions but the AD. Another cell type involved in the neuroinflammatory process is the astrocyte marked by GFAP expression [57]. Tissue levels of GFAP were significantly elevated in all brain regions of injured animals. However, the change in GFAP levels was the most robust suggesting that astroglial response (i.e., gliosis) is a more global response in the injured brain than some of the markers we as well as others have analyzed. This global glial response to injury is consistent with the numerous and somewhat diverse functions astroglia play in the normal and injured CNS [58-62].

Of the markers related to metabolic changes/oxidative stress and vascular functions, only vWF levels were not significantly increased in injured rats relative to their sham counterparts. This finding is in contrast with the significant increases in vWF levels in the PFC of injured rats compared to both, naïve and sham animals. However, this supports our previous findings that experimental manipulations alone (i.e., sham conditions) can trigger pathological changes due to the role of stress as a cofactor in bTBI [11, 14]. These findings also suggest that some of the changes can be spatially/anatomically restricted and may not be reflected in the systemic circulation. This notion is supported by the finding that plasma levels of several inflammatory markers (MIP1 α , CCR5, MCP1 and CRP) were unchanged while there were significant changes in their tissue levels, again mostly in the PFC. As discussed above, these markers are involved in mediating the injury process at various levels.

Elevated plasma levels of the measured cell surface markers (integrin $\alpha 6$, TIMP1 and TIMP4) are likely indicative of the increases measured in brain tissue. All brain-specific proteins were significantly elevated in the plasma of injured animals relative to sham animals. These increases in neuronal and glial markers suggest complex neuropathologies that include glial loss/damage as indicated by elevated GFAP and S100 β levels. Similarly, increased NSE, NF-H and CK-BB levels reflect neuronal damage and/or loss. One of the most substantive increases in plasma biomarker levels was of tau protein. This finding may be especially significant as tau pathologies have been suspected to play a key role in the development of chronic neurodegenerative conditions (e.g., chronic traumatic encephalopathy) following rmTBI [63-68].

In summary, we found that: 1) ICV in the rodent model of blast-induced rmTBI is measured in hours, which roughly translates to 1-2 weeks in humans. 2) A number of complex molecular pathologies contribute to ICV in blast-induced rmTBI; these include metabolic changes/oxidative stress, vascular dysfunction, altered cellular adhesion and interaction, inflammation, as well as neuronal and glial damage and/or loss. 3) The molecular pathologies of which virtually all affected the PFC may potentially account for the increased anxiety- and depression-related behaviors exhibited by injured rats; this observation maybe related to the PFC's anatomical localization and vulnerability to physical injuries, especially if they are repetitive in nature. 4) These pathologies are also reflected in altered plasma levels of biomarkers that can be validated in humans and developed into blood-based diagnostics for the purpose of identifying individuals with ICV. A limitation of our current study is that injury-induced changes in marker plasma levels were determined at a single, terminal time point. Accordingly, serial blood sampling would provide invaluable information about the temporal pattern of molecular changes that may be critical for the identification of the period of ICV in rmTBIs.

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Animal handling and treatments were conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations related to animals and experiments involving animals, and adhered to principles stated in the Guide to the Care and Use of Laboratory Animals, National Research Council. The facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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Figure captions

Fig. 1. Significant albeit transient reductions in arterial oxygen saturation and heart rate were measured in injured rats. **(A)** arterial oxygen saturation levels (%), **(B)** heart rate (number of beats per min), **(C)** pulse distension (μm), and **(D)** breath rate (number of breaths per min) were obtained under isoflurane anesthesia at baseline, immediately following injury (x5 for injured rats), and at 24 h post-injury. Data are presented as the mean \pm S.E.M. $^{\$}p < 0.05$ for sham pre- vs. post-injury values; $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.01$ for injured pre- vs. post-injury values.

Fig. 2. Injured rats exhibit a decline in gross movement and an elevation in depression- and anxiety-related behaviors. An open field system was used to measure **(A)** horizontal activity (number of beam breaks), **(B)** vertical activity (number of beam breaks), and **(C)** center time (seconds) at baseline and 24 h following blast (or sham) exposure. Data are presented as the mean \pm S.E.M.

Fig. 3. Brain tissue levels of oxidative stress and vascular protein biomarkers in the selected brain regions. Tissue extracts were prepared from the prefrontal cortex (PFC), amygdala (AD), dorsal and ventral hippocampus (DHC and VHC, respectively) of naïve, sham, and injured rats. The reported Y-cept values (log10) indicate relative protein concentrations; data are presented as the mean \pm S.E.M. $^*p < 0.05$ and $^{**}p < 0.01$ for naïve vs. injured; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ for sham vs. injured.

Fig. 4. Brain tissue levels of proteins related to cellular adhesion, extracellular matrix, and inflammation in the selected brain regions. Tissue extracts were prepared from the prefrontal cortex (PFC), amygdala (AD), dorsal and ventral hippocampus (DHC and VHC, respectively) of naïve, sham, and injured rats. The reported Y-cept values (log10) indicate relative protein concentrations; data are presented as the mean \pm S.E.M. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for naïve vs. injured; $^{\$}p < 0.05$ and $^{\$\$}p < 0.01$ for sham vs. injured.

Fig. 5. Brain tissue levels of protein biomarkers related to inflammation and glial cell response in the selected brain regions. Tissue extracts were prepared from the prefrontal cortex (PFC), amygdala (AD), dorsal and ventral hippocampus (DHC and VHC, respectively) of naïve, sham, and injured rats. The reported Y-cept values (log10) indicate relative protein concentrations; data are presented as the mean \pm S.E.M. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for naïve vs. injured; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, and $^{\$\$\$}p < 0.001$ for sham vs. injured.

Table captions

Supplementary Table. Antibody names, origins, and respective dilutions for use in reverse phase protein microarray.

Table 1. Plasma concentrations of oxidative stress and vascular protein biomarkers two days after blast overpressure (or sham) exposure

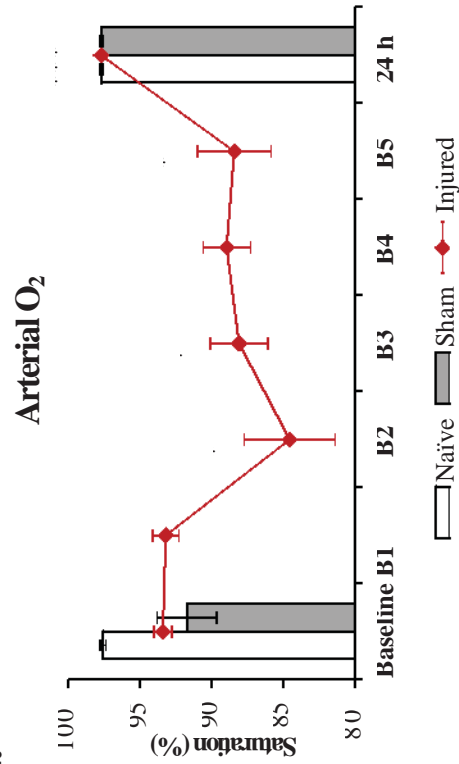
Table 2. Plasma concentrations of cellular adhesion and extracellular matrix protein biomarkers two days after blast overpressure (or sham) exposure

Table 3. Plasma concentrations of inflammatory protein biomarkers two days after blast overpressure (or sham) exposure

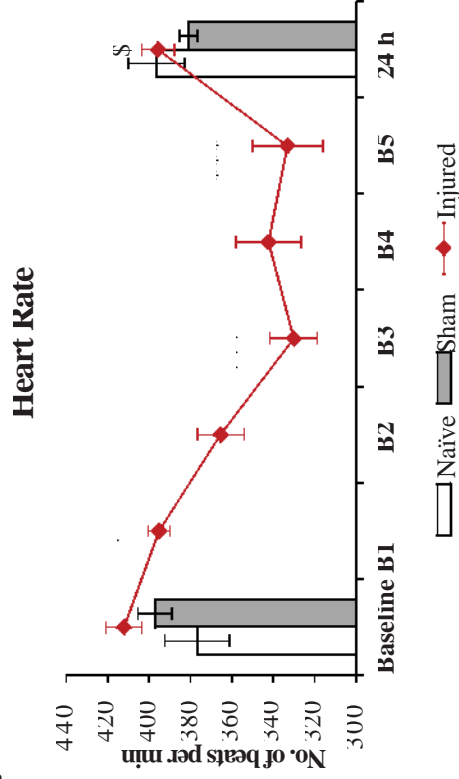
Table 4. Plasma concentrations of glial and neuronal protein biomarkers two days after blast overpressure (or sham) exposure

Figure 1

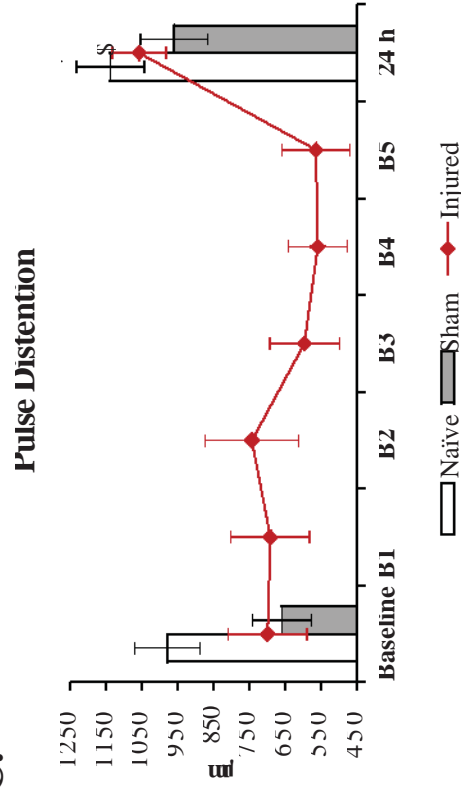
A.



B.



C.



D.

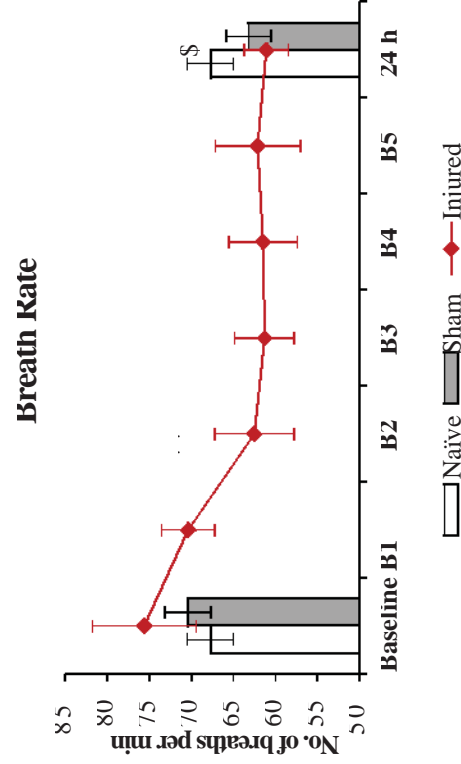
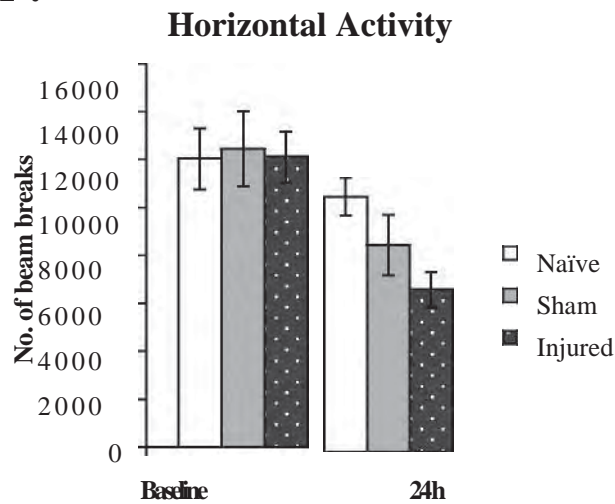


Figure 2

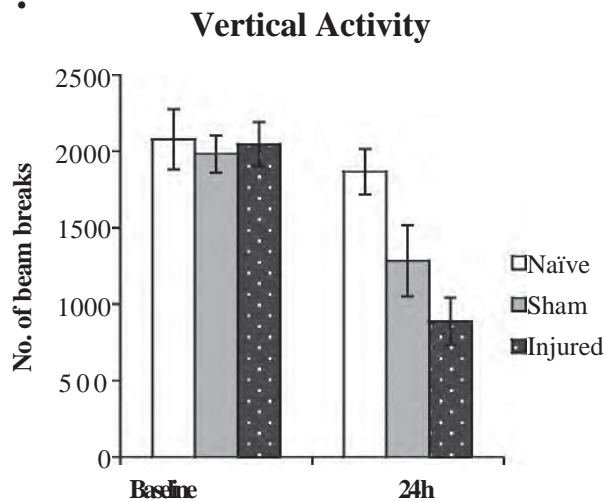
A .



ANOVA

Baseline: not significant
24 h (covaried by baseline): *main effect for Condition*
 $F(2, 17) = 4.75, p = 0.023, \eta^2 = 0.359$
Naïve vs. Injured (pairwise comparison, $p = 0.008$)

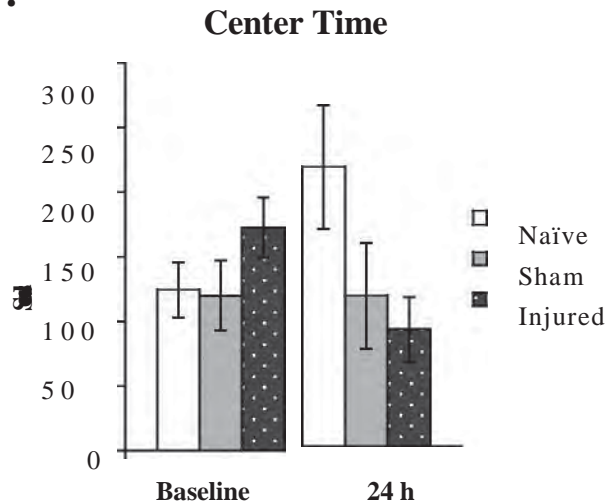
B .



ANOVA

Baseline: not significant
24 h (covaried by baseline): *main effect for Condition*
 $F(2, 17) = 5.49, p = 0.014, \eta^2 = 0.393$
Naïve vs. Injured (pairwise comparison, $p = 0.005$)

A .



ANOVA

Baseline: not significant
24 h (covaried by baseline): *main effect for Condition*
 $F(2, 17) = 7.36, p = 0.005, \eta^2 = 0.464$
Naïve vs. Injured (pairwise comparison, $p = 0.001$)
Naïve vs. Sham (pairwise comparison, $p = 0.054$)

Figure 3

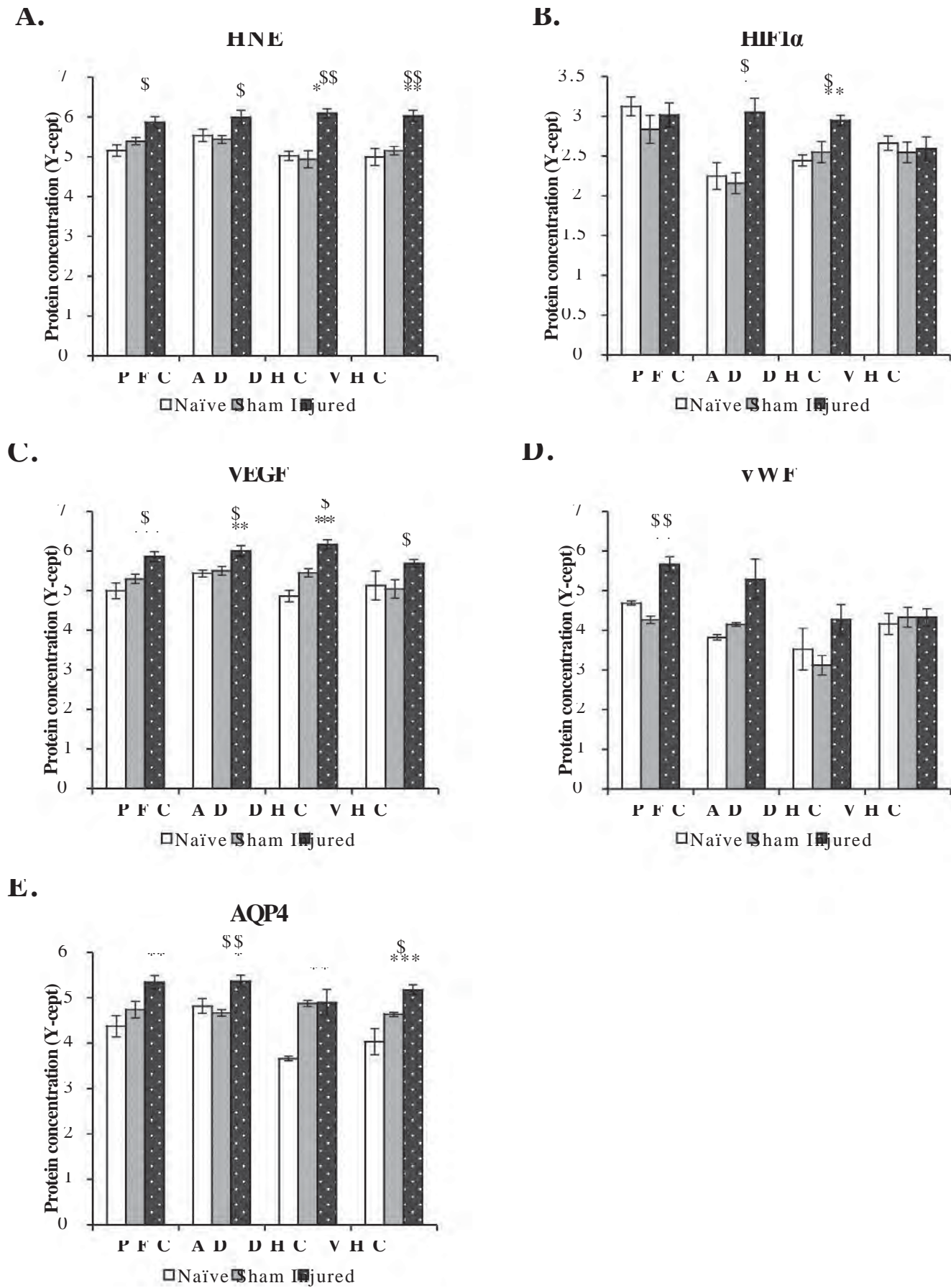
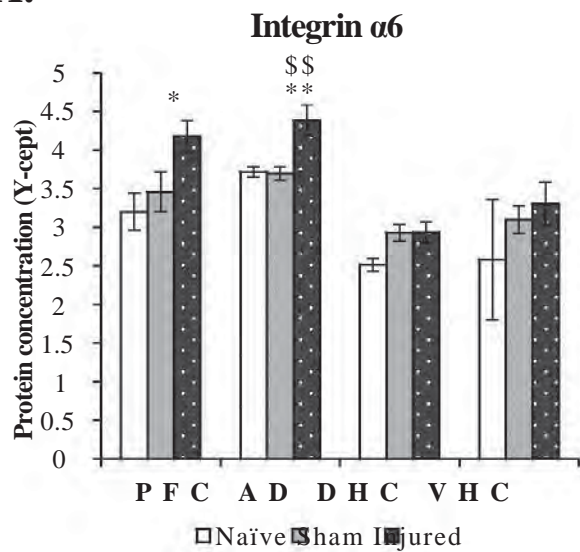
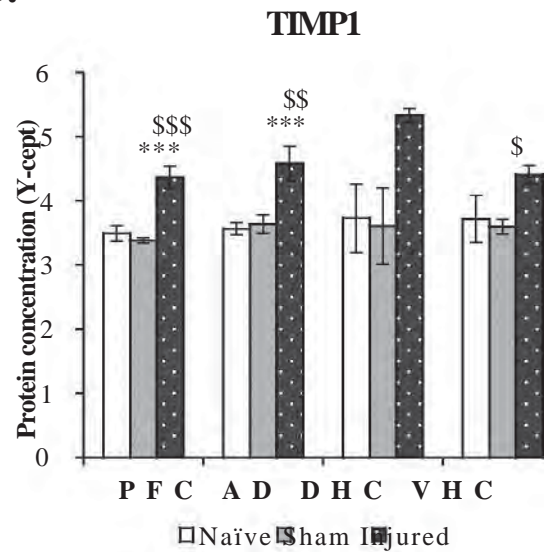


Figure 4

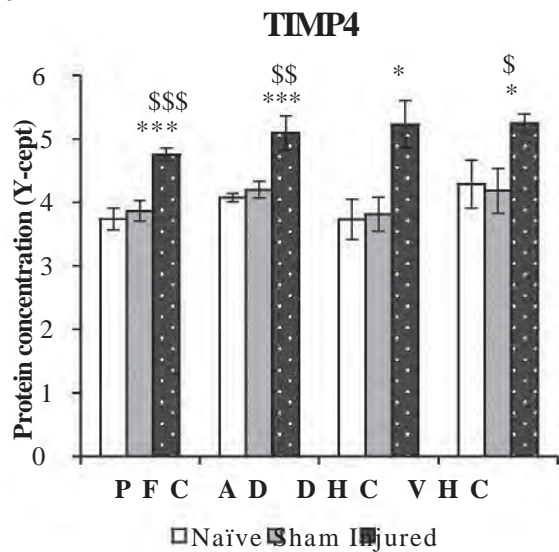
A.



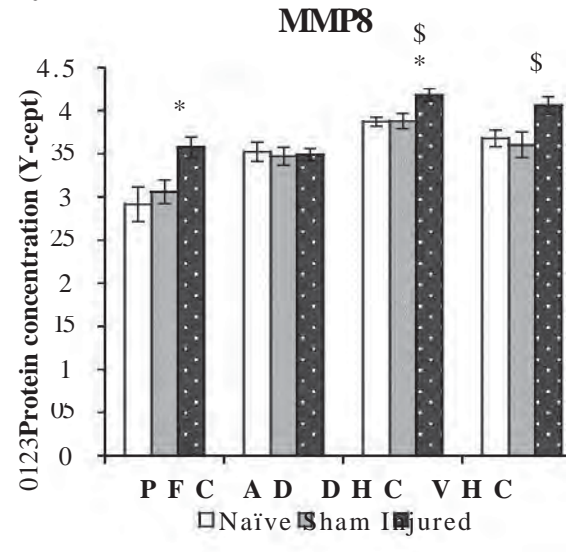
B.



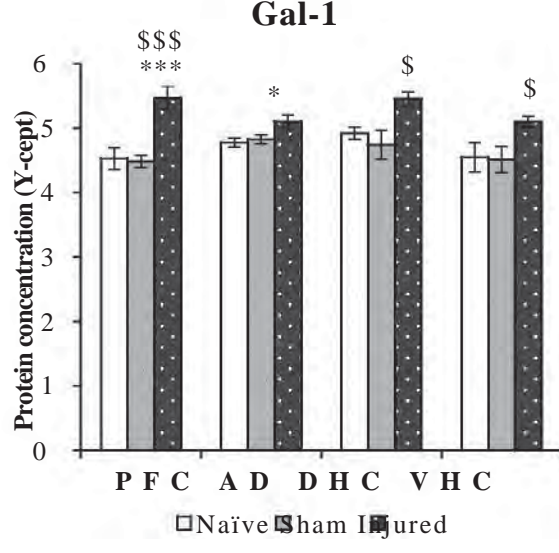
C.



D.



E.



F.

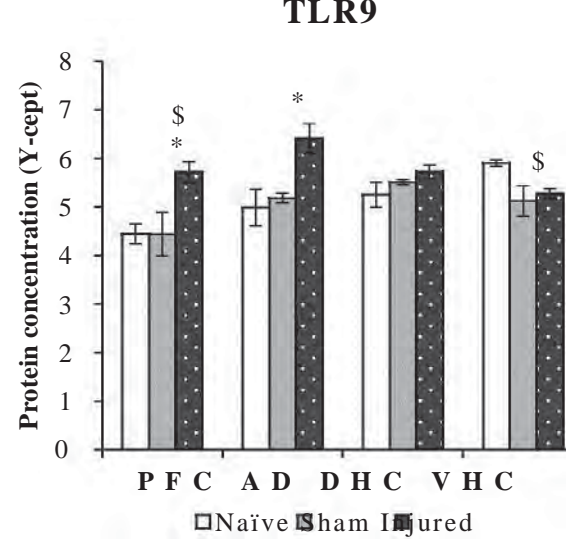
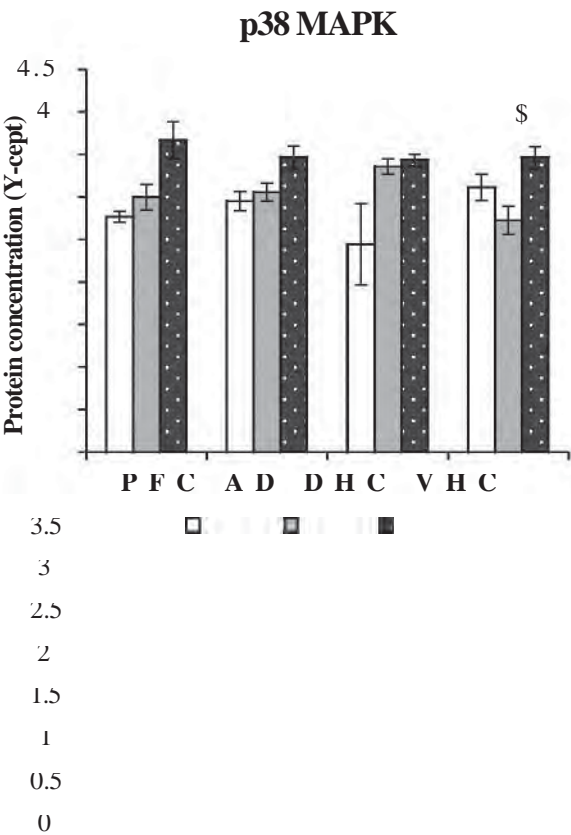
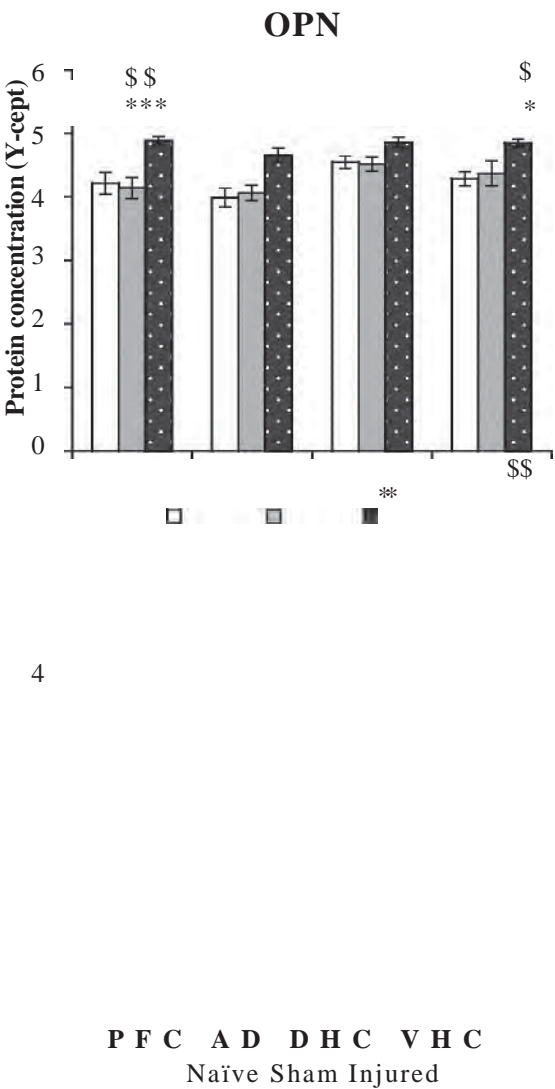


Figure 5

A.



B.

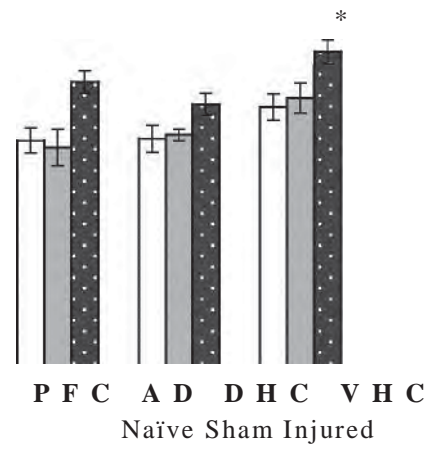


C.

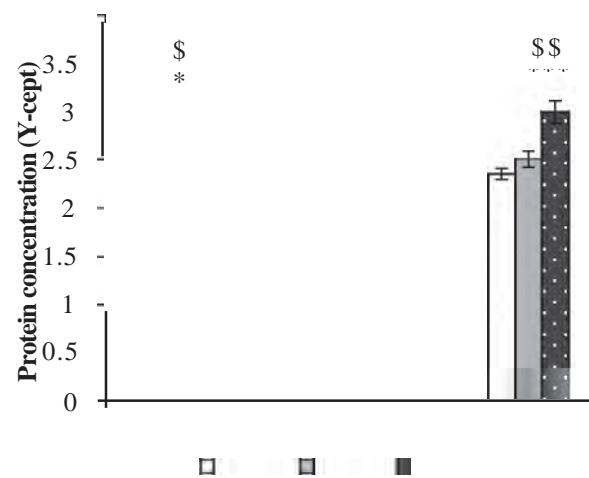
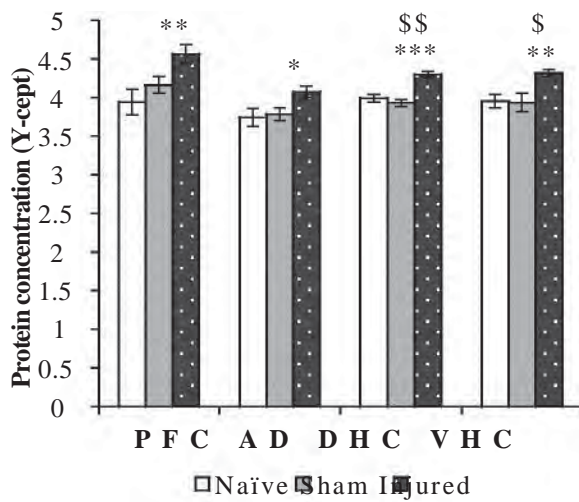
MIP1 α

D.

CCR5

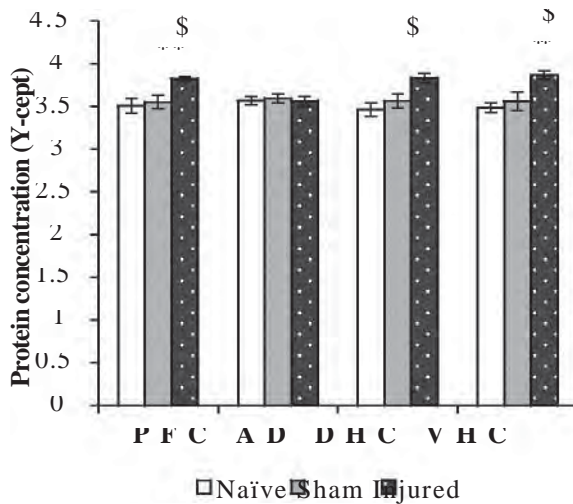


GFAP

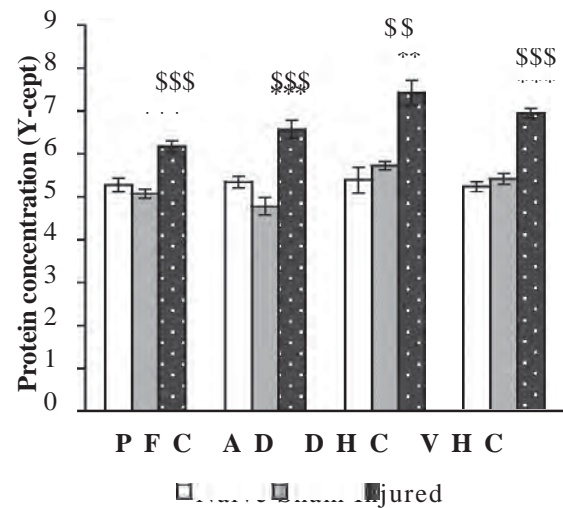


E.

CD53 (OX44)



F.



Antibody	Vendor	Catalog No.	Dilution
Aquaporin 1 (AQP1)	Abcam	ab87845	50
Aquaporin 4 (AQP4)	Abcam	ab128906	50
CD53 (OX44)	GeneTex	GTX76102	20
Ceruloplasmin	GeneTex	GTX28813	20
Chemokine (C-C motif) receptor 5 (CCR5)	GeneTex	GTX61751	20
Claudin 5	Santa Cruz	sc-28670	20
C-reactive protein (CRP)	Santa Cruz	sc-30047	20
Creatine kinase-brain type (CK-BB)	Santa Cruz	sc-15157	20
Cytokine-induced neutrophil chemoattractant 1 α (CINC1 α)	GeneTex	GTX10365	15
Fetal liver kinase 1 (FLK1/VEGF receptor 2)	Santa Cruz	sc-315	20
Fibrinogen	Santa Cruz	sc-69775	20
N-formyl peptide receptor (FPR)	Santa Cruz	sc-13198	20
Galectin-1 (Gal-1)	GeneTex	GTX62666	20
Glial fibrillary acidic protein (GFAP)	Abcam	ab7260	500
4-Hydroxy-2-noneal (HNE)	Calbiochem	393207	100
Hypoxia-inducible factor 1 α (HIF1 α)	Santa Cruz	sc-53546	20
Integrin α 6	GeneTex	GTX100565	100
Macrophage inflammatory protein 1 α (MIP1 α)	Santa Cruz	sc-166911	20
Matrix metalloproteinase 8 (MMP8)	Santa Cruz	sc-50384	20
Monocyte chemotactic protein 1 (MCP1)	Santa Cruz	sc-1784	20
Neurofilament-heavy chain (NF-H)	Sigma-Aldrich	N4142	20
Neuron-specific enolase (NSE)	Abcam	ab53025	50
Osteopontin (OPN)	Santa Cruz	sc-73631	20
p38 Mitogen-activated protein kinase (p38 MAPK)	Cell Signaling	9212	100
S100 calcium binding protein β (S100 β)	Abcam	ab41548	20
Tau	Santa Cruz	sc-1995	20
TIMP metalloproteinase inhibitor 1 (TIMP1)	GeneTex	GTX108254	100
TIMP metalloproteinase inhibitor 4 (TIMP4)	GeneTex	GTX114942	100
Toll-like receptor 9 (TLR9)	Santa Cruz	sc-13218	20
Vascular endothelial growth factor (VEGF)	Abcam	ab53465	50
von Willebrand factor (vWF)	Santa Cruz	sc-8068	20

Table 1. Oxidative stress and vascular protein biomarker levels in the plasma

	Group	Mean \pm S.E.M.	ANOVA		Comparison of Means			
			<i>F</i> value	<i>p</i> value	Naïve vs. Injured		Sham vs. Injured	
					Mean diff.	<i>p</i> value	Mean diff.	<i>p</i> value
HNE	Naïve	6.355 \pm 0.12	12.769	0.000	-0.508	0.000	-0.399	0.004
	Sham	6.464 \pm 0.08						
	Injured	6.863 \pm 0.06						
HIF1 α	Naïve	4.289 \pm 0.30	6.303	0.006	-0.986	0.012	-0.878	0.026
	Sham	4.397 \pm 0.21						
	Injured	5.275 \pm 0.19						
Ceruleplasmin	Naïve	6.469 \pm 0.04	19.622	0.000	-0.485	0.000	-0.375	0.000
	Sham	6.579 \pm 0.04						
	Injured	6.955 \pm 0.07						
VEGF	Naïve	5.793 \pm 0.08	9.664	0.001	-0.918	0.004	-0.998	0.003
	Sham	5.713 \pm 0.03						
	Injured	6.711 \pm 0.23						
vWF	Naïve	4.095 \pm 0.14	6.014	0.009	-0.684	0.010	-0.539	0.067
	Sham	4.239 \pm 0.12						
	Injured	4.779 \pm 0.17						
AQP1	Naïve	5.119 \pm 0.26	6.050	0.007	-0.902	0.016	-0.851	0.023
	Sham	5.169 \pm 0.25						
	Injured	6.021 \pm 0.15						
AQP4	Naïve	6.037 \pm 0.05	12.450	0.000	-0.413	0.000	-0.335	0.004
	Sham	6.114 \pm 0.06						
	Injured	6.450 \pm 0.07						
FLK1	Naïve	4.868 \pm 0.17	8.117	0.002	-0.745	0.003	-0.576	0.023
	Sham	5.036 \pm 0.08						
	Injured	5.612 \pm 0.14						
Claudin 5	Naïve	5.221 \pm 0.12	11.453	0.000	-0.797	0.000	-0.482	0.031
	Sham	5.537 \pm 0.07						
	Injured	6.018 \pm 0.13						

Mean protein values (log10) measured in the plasma of naïve, sham, and injured rats. Significant differences in protein biomarker levels are indicated in boldface.

Table 2

Table 2. Cell adhesion and extracellular matrix protein biomarker levels in the plasma

		Comparison of Means						
		ANOVA			Naïve vs. Injured		Sham vs. Injured	
	Group	Mean ± S.E.M.	F value	p value	Mean diff.	p value	Mean diff.	p value
Integrin α6	Naïve	4.209 ± 0.12	18.567	0.000	-1.379	0.000	-1.363	0.000
	Sham	4.224 ± 0.20						
	Injured	5.587 ± 0.19						
TIMP1	Naïve	4.178 ± 0.18	9.777	0.001	-1.439	0.001	-1.043	0.019
	Sham	4.574 ± 0.25						
	Injured	5.617 ± 0.27						
TIMP4	Naïve	4.650 ± 0.08	14.195	0.000	-0.564	0.000	-0.460	0.002
	Sham	4.753 ± 0.07						
	Injured	5.213 ± 0.09						

Mean protein values (log10) measured in the plasma of naïve, sham, and injured rats. Significant differences in protein biomarker levels are indicated in boldface.

Table 3

Table 3. Inflammatory protein biomarker levels in the plasma

	Group	Mean \pm S.E.M.	ANOVA		Comparison of Means			
			<i>F</i> value	<i>p</i> value	Naïve vs. Injured		Sham vs. Injured	
					Mean diff.	<i>p</i> value	Mean diff.	<i>p</i> value
Galectin	Naïve	5.079 \pm 0.05	6.134	0.007	-0.286	0.040	-0.354	0.010
	Sham	5.011 \pm 0.11						
	Injured	5.365 \pm 0.07						
p38 MAPK	Naïve	3.732 \pm 0.39	8.502	0.002	-1.396	0.005	-1.317	0.008
	Sham	3.811 \pm 0.29						
	Injured	5.128 \pm 0.20						
MIP1 α	Naïve	4.690 \pm 0.16	4.513	0.024	-0.371	0.042	-0.353	0.067
	Sham	4.707 \pm 0.09						
	Injured	5.060 \pm 0.06						
CCR5	Naïve	4.026 \pm 0.20	5.170	0.018	-0.896	0.017	-0.586	0.163
	Sham	4.337 \pm 0.13						
	Injured	4.922 \pm 0.22						
MCP1	Naïve	4.053 \pm 0.09	6.449	0.006	-0.552	0.006	-0.387	0.060
	Sham	4.218 \pm 0.11						
	Injured	4.605 \pm 0.13						
CINC1 α	Naïve	3.860 \pm 0.06	37.765	0.000	-1.224	0.000	-1.337	0.000
	Sham	3.746 \pm 0.11						
	Injured	5.083 \pm 0.14						
Fibrinogen	Naïve	4.840 \pm 0.22	6.932	0.005	-0.722	0.007	-0.595	0.038
	Sham	4.968 \pm 0.09						
	Injured	5.563 \pm 0.13						
CRP	Naïve	5.154 \pm 0.17	3.864	0.036	-0.474	0.040	-0.362	0.156
	Sham	5.266 \pm 0.08						
	Injured	5.628 \pm 0.12						
FPR	Naïve	4.831 \pm 0.31	4.166	0.038	-0.498	0.447	-0.917	0.031
	Sham	4.412 \pm 0.36						
	Injured	5.329 \pm 0.08						

Mean protein values (log10) measured in the plasma of naïve, sham, and injured rats. Significant differences in protein biomarker levels are indicated in boldface.

Table 4

Table 4. Glial and neuronal protein biomarker levels in the plasma

			Comparison of Means					
	Group	Mean ± S.E.M.	ANOVA		Naïve vs. Injured		Sham vs. Injured	
			F value	p value	Mean diff.	p value	Mean diff.	p value
GFAP	Naïve	4.725 ± 0.11	8.785	0.001	-0.556	0.010	-0.673	0.003
	Sham	4.608 ± 0.13						
	Injured	5.281 ± 0.13						
CD53 (OX44)	Naïve	4.612 ± 0.21	3.787	0.037	-0.411	0.180	-0.587	0.038
	Sham	4.436 ± 0.16						
	Injured	5.024 ± 0.12						
S100β	Naïve	4.163 ± 0.11	68.988	0.000	-1.264	0.000	-1.460	0.000
	Sham	3.967 ± 0.08						
	Injured	5.428 ± 0.10						
NSE	Naïve	3.541 ± 0.09	26.101	0.000	-1.129	0.000	-1.076	0.000
	Sham	3.594 ± 0.06						
	Injured	4.670 ± 0.15						
NF-H	Naïve	5.732 ± 0.05	6.310	0.006	-0.196	0.033	-0.241	0.011
	Sham	5.687 ± 0.05						
	Injured	5.928 ± 0.05						
CK-BB	Naïve	4.342 ± 0.14	6.930	0.004	-0.568	0.007	-0.484	0.023
	Sham	4.426 ± 0.11						
	Injured	4.910 ± 0.11						
Tau	Naïve	4.454 ± 0.14	30.884	0.000	-1.635	0.000	-1.549	0.000
	Sham	4.540 ± 0.27						
	Injured	6.089 ± 0.15						

Mean protein values (log10) measured in the plasma of naïve, sham, and injured rats. Significant differences in protein biomarker levels are indicated in boldface.



Blast exposure phosphorylates *Tau* preferentially at serine396, which can trigger Alzheimer's-like pathology

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Abstract

Blast-induced traumatic brain injury (TBI) is one of the major disabilities in service members returning from recent military operations. Blast-induced TBI is associated with acute and chronic neuropathological and neurobehavioral deficits. Epidemiological studies indicate that brains of 30% of victims who die acutely following TBI have A β plaques, a pathological feature of Alzheimer's disease (AD), which suggests that TBI may predispose to AD, although to date his notion remains somewhat speculative. *Tau* protein, phosphorylated at Serine396 (S396), is rich in paired helical filaments which form neurofibrillary tangles (NFTs) observed in the brains of patients with AD. The number of NFTs is tightly linked to the degree of dementia, indicating that the formation of NFTs may underlie and contribute to neuronal dysfunction. Preliminary studies carried out in our laboratory using shock tube models of single and repeated blast-induced TBI in rats indicate that phosphorylation of *Tau* protein occurs preferentially at S396. Phosphorylation of *Tau* varied in different regions of the brain and the degree of phosphorylation increased with number of blast exposures in many of the brain regions. Increased phosphorylation of *Tau* occurred acutely after blast exposures and chronically returned towards normal levels which at this stage did not positively correlate with the accumulation of amyloid precursor protein (APP) that occurred chronically. These results indicate that acute *Tau* protein phosphorylation at S396 and chronic accumulation of APP in the brain after blast exposure may predispose to Alzheimer's-like neuropathology.

Background

The complex biochemical and molecular mechanisms of blast-induced TBI and how they trigger subsequent secondary pathological processes and behavioral deficits are not well understood. Brain injury severity after blast exposure was found to increase with number of blast exposures. Studies indicate accumulation of A β plaques in the brains of TBI victims suggesting that TBI may predispose to Alzheimer's-like pathology. The NFTs present in the brains of patients with AD is rich in *pTau*(S396) indicating a potential role of phosphorylation of *Tau* at Serine396 in the pathology of AD. In the present study, we explored the phosphorylation of *Tau* protein and accumulation of APP in the brain after single and double blast exposures using the shock tube model of blast-induced TBI in rats.

Methods

Animals: Male Sprague Dawley rats (300-350g)

Blast TBI: After anesthesia (isoflurane, 4%), animals were secured in a prone position in the shock tube and exposed to single (19psi) or double (two 19psi blasts within 1 min) blast exposures.



Animal holder



Western blotting: At different intervals after blast exposures, brain tissues were collected for Western blotting for total *pTau*, APP, *pTau*(S396), *pTau*-T231 and β -actin. Band intensities were determined by densitometry.

Results

Blast exposure increased the phosphorylation of *Tau* protein in most of the brain regions in a time dependent manner with maximum increase at early stages. The degree of phosphorylation varied in different brain regions. The phosphorylation of *Tau* protein increased with number of blast exposures.

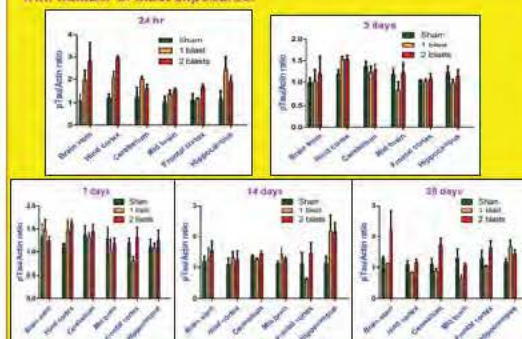


Fig.1. Expression of total phosphorylated *Tau* proteins in different brain regions after blast exposure

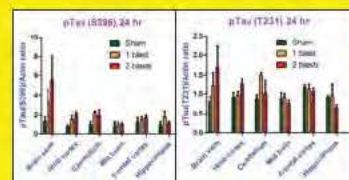


Fig.2. Expression of *pTau*(S396) and *pTau*(T231) proteins in different brain regions after blast exposure

After blast exposure, phosphorylation of *Tau* at Serine396 was higher than at Threonine231

Results continued.....

Blast exposure increased the accumulation of APP in most of the brain regions in a time-dependent manner with maximum accumulation at chronic stages. APP accumulation varied in different regions of the brain. The accumulation of APP increased with the number of blast exposures in most of the brain regions.

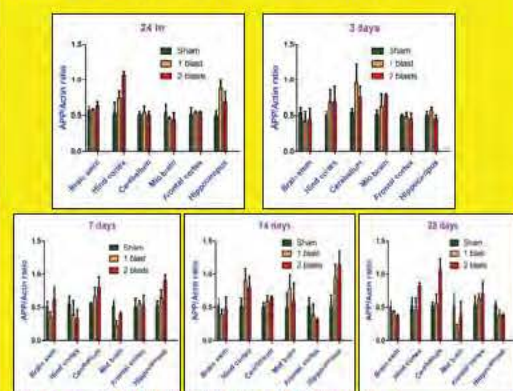


Fig.3. Expression of APP in different brain regions after blast exposure

Summary/Discussion

- ♦ Blast exposure caused phosphorylation of *Tau* protein in different brain regions in a time dependent manner. Maximum phosphorylation of *Tau* proteins occurs at early stages of injury. The phosphorylation of *Tau* protein was found to increase with repeated blast exposures in many brain regions. The degree of phosphorylation of *Tau* protein after blast exposure varied in different regions of the brain.
- ♦ The phosphorylation of *Tau* protein at Serine396 was found to be higher than that at Threonine231. The preferential phosphorylation of *Tau* protein at Serine396 may be playing a role in Alzheimer's-like pathology observed in TBI victims.
- ♦ Blast exposure results in the accumulation of APP in different brain regions in a time-dependent manner with maximum accumulation at chronic stages. Accumulation of APP was found to be increasing with the number of blast exposures.
- ♦ Phosphorylation of *Tau* protein didn't show a positive correlation with the accumulation of APP in different brain regions suggests a distinct pathological mechanism leading to Alzheimer's-like neuropathology after blast exposure.

Acknowledgements

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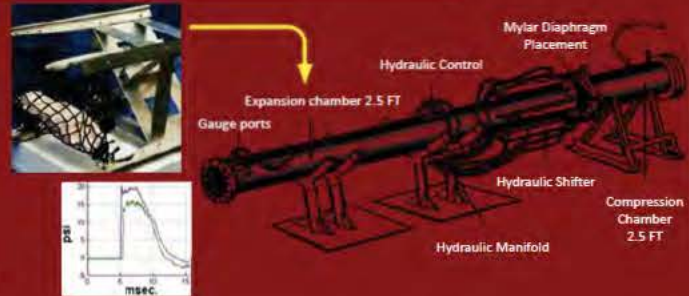


Brain Pathological and Biochemical Responses Following Repeated Blast Exposures in Rats

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Introduction and Methods

- Pathophysiological sequelae of blast-induced traumatic brain injury (TBI) are mediated by an interaction of acute and delayed molecular, cellular and physiological events.
- CCL2 and its receptor CCR2 have been shown to be involved in various neurodegenerative disorders, prompting speculation that they could also contribute to progressive neurodegeneration following blast TBI.
- Isoflurane anesthetized Sprague Dawley rats, male, 350 ± 25 g were exposed to blast overpressure in an air-driven shock tube.
- Two closely coupled blast shockwaves (peak total pressure of 23 psi, 8 msec positive phase duration) separated by 30 sec were employed to compare to a single blast exposure.
- Brain tissue underwent morphological assessment, protein measurement and gene expression analysis by using silver staining, immunohistochemistry, ELISA and qRT-PCR technologies.



Results

Fig. 1. The level of CCL2 in CSF. ELISA data showed that CCL2 increased significantly in CSF at 6 h and 24 h post-injury ($n = 5$, each group). Compared to single BOP, double blasts caused remarkable higher level of CCL2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

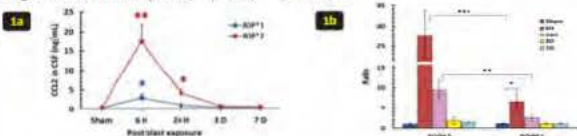


Fig. 2. The level of CCL2 in plasma. Plasma CCL2 level increased significantly at 6 h after single and double blast exposure ($n = 5$) * $p < 0.05$, ** $p < 0.01$.

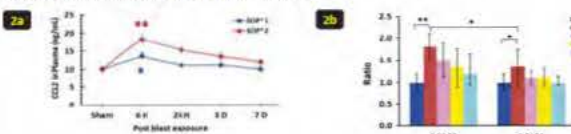


Fig. 3. The level of CCL2 in brain regions. CCL2 can be released from neurons and glia. The distribution of CCL2 in cerebellum (Cb), brainstem (Bs), cortex (ct) and hippocampus (Hip) was demonstrated in 3a and 3b. ELISA data also showed that CCL2 increased significantly in multiple brain regions in single (3c) and double blast (3d) injured rats. For each group $n = 5$, * $p < 0.05$.

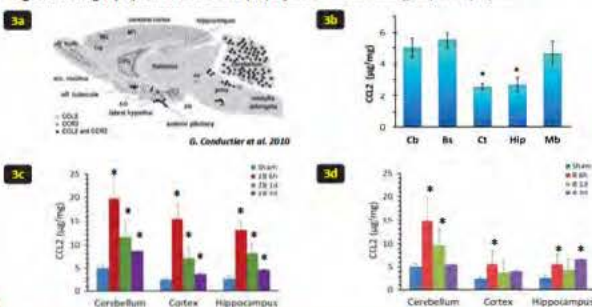


Fig. 4. Blast induced gene changes. Cerebellum at 6 h post double BOPs was analyzed by qPCR. The results indicated that blast exposures elicited DNA damage, inflammation and neural growth associated gene expression changes.



Fig. 5. Blast induced axonal degeneration. The 4%PFA fixed brain sections were processed using Neurosilver kit. Images demonstrated high dense precipitation in the sections of cortex (5a) and cerebellum (5b) at 3 d, 7 d and up to 90 days (not shown) post blast exposures.



Fig. 6. Blast induced glial cells proliferation. Brain sections were incubated with primary antibodies of GFAP (6a, green) and Iba1 (6b, red) for verification of astrocytes and microglial cells, respectively.

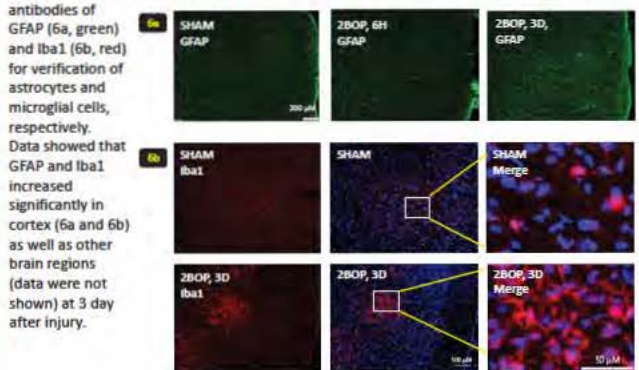
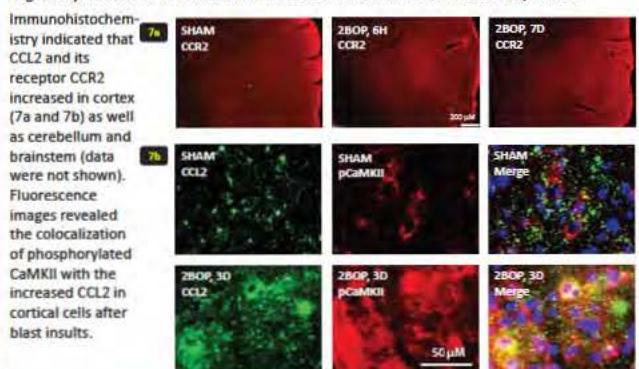


Fig. 7. Expressions of CCL2 and CCR2 in cerebral cortex after double blast exposures.



Conclusions

- An inflammatory response represents one of the first immune processes following injury. CCL2 level in CSF increased in a blast overpressure dependent manner that can be a dosimeter for blast TBI.
- Significantly increased clustered phosphorylated CaMKII indicate that blast exposure causes hyper neuronal network activity which may lead to neuronal excitatory toxicity, while increased phosphorylation of Tau and eIF2 α may promote apoptosis and leading to the chronic traumatic encephalopathy.
- Compared to a single blast, double blast-induced TBI in rats model demonstrated significant pathological and biochemical changes in CNS that can be used for therapeutic drug development and evaluation.

DISCLAIMER There is no objection to its presentation. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals.

Characterization of Blast-Induced Auditory and Vestibular Injury in Rats

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ABSTRACT Blast exposure is the most common cause of traumatic brain injury (TBI) in warfighters. Nearly 60% of blast TBI victims exhibit hearing loss, tinnitus, dizziness and balance disorders. To date, the etiologies of these injuries are largely undefined. A high fidelity animal model is critical to define the mechanism(s) of injury and develop therapeutic strategies for blast-induced neurobehavioral deficits. In this study, we used an air-driven shock tube to simulate primary blast and investigated the pathological effects of blast exposures on central and peripheral auditory/vestibular systems. Anesthetized rats were tautly secured in a transverse prone position 2.5 ft within the mouth of a 1 ft diameter shock tube with the right side facing the oncoming shockwave. Rats were exposed to two closely coupled shockwaves (peak total pressures of 5, 12 or 19 psi) separated by 30 sec. Rats were euthanized at varied intervals (6 h, 24 h, 7 d and 14 d) post injury and tissues underwent histological and RNA analyses. All rats received rotarod training prior to and testing after blast exposures for evaluation of motor coordination and balance. Compared to a single blast insult, repeated blast exposures significantly impaired motor coordination. Intensity-dependent blast-induced damage to middle and inner ears was evident with no significant differences between left and right ears. Labyrinthine hemorrhage was prominent at 24 h up to 14 days after blast exposures. Repeated blast exposures caused significant axonal degeneration and glial cell proliferation in the central vestibular signal processing regions of the brain, also triggered multiple gene expression changes that are associated with DNA repair, neural growth, inflammation and pain. These findings indicate that both peripheral and central vestibular systems are vulnerable to blast injuries, and are particularly disrupted by closely coupled repeated blasts. Neuroinflammation, which occurred during the early phase post injury, could be a major factor leading to secondary neuronal damage.

METHODS

Animals and TBI model: Sprague Dawley rats, male, 350 ± 25 g (Charles River Laboratories), were anesthetized with isoflurane and were tautly secured in a transverse prone position 2.5 ft within the mouth of a 1 ft diameter shock tube with the right side of the animal facing the oncoming shockwave. Rats were exposed to two closely coupled shockwaves (peak total pressures of 5, 12 or 19 psi) separated by 30 sec. Rats were euthanized at varied intervals post-injury and tissues underwent histological and RNA analyses.

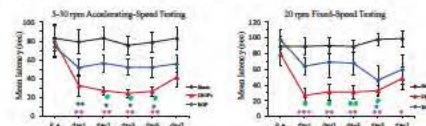
Neurobehavioral Assessment: Rotarod balance testing was used to assess the ability of an animal to balance on a rotating rod. Rats received fixed speed and accelerating-speed rotarod training for 5 days prior to blast exposure and were tested at 1, 2, 3, 5 and 7 days post-injury. One way ANOVA and a Student-Newman-Keuls were used for data analysis.

Histopathological Analysis: Rats were fully anesthetized and perfused with saline followed by 4% paraformaldehyde. Frozen brain tissues were cut in coronal sections (30 μ m). The sections were processed using Neurosilver kit (FD Neurotechnologies). Following 0.2% Triton X-100 solution permeabilization and blocking with 10% Goat serum blocking, brain sections were incubation with primary antibodies (GFAP or CCR2) overnight at 4°C. After incubated with Alexa Fluor-conjugated secondary antibody, sections were mounted in SlowFade gold antifade reagent with DAPI (Invitrogen). Decalcification in 0.12 M EDTA in 0.1 M PB (pH 7.0) for 5 days, ear tissues were embedded into paraffin and cut in longitudinal sections (10 μ m) of the cochlea. Serial sections were stained with hematoxylin and eosin (H&E). All images were examined under AX-80 Olympus microscope.

RNA extraction and qRT-PCR: Tissue samples were homogenized in ice cold TRIzol (Life Technologies), and RNA was isolated following the TRIzol protocol. RNA purity was determined using NanoDrop ND-1000 (Thermo Fisher). According to the manufacturer's instructions, 800 ng of high-quality RNA for each sample was converted into cDNA and applied to PCR array plates using the RT2 PCR array kit (QIAGEN). All reactions were processed in the 7500 Fast Real-Time PCR System (Life Technologies). Data were interpreted by using QIAGEN's web-based PCR array data analysis tool.

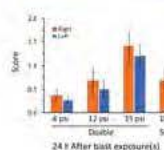
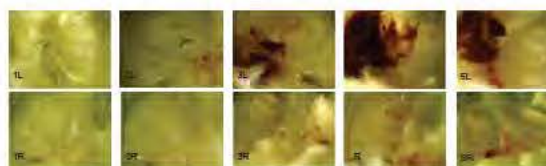
RESULTS

1 Functional deficit after blast exposure



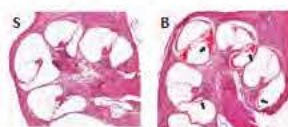
- Rat's performance on the rotarod before and post-injury
- Experimental groups: Sham (without blast), BOP (single 19 psi), 2BOPs (double 19 psi), 7 rats each group
- 2 BOPs vs. sham: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$; 2 BOPs vs. BOP, @ $p < 0.05$, @@ $p < 0.01$; BOP vs. sham, * $p < 0.05$, *** $p < 0.01$

2 Effect of blast exposure(s) on middle and inner ears



- 24 h after blast exposure, temporal bones were removed and tympanic bullae were opened
- Images were taken under microscope with a 20x magnification
- Sham (1L, 1R), 2BOPs_5 psi (2L, 2R), 2BOPs_12 psi (3L, 3R), 2BOPs_19 psi (4L, 4R), BOP_19 psi (5L, 5R)

3 Inner ear hemorrhage after blast exposures



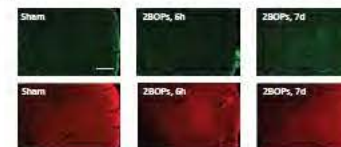
- 14 d after blast exposures
- Sham (S), 2BOPs_19psi (B)
- Cross sections with H & E staining
- Original magnification: x 200
- Hemorrhage (arrow)

4 Effect of blast exposure on cerebellum and brainstem



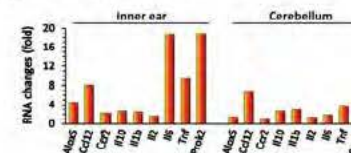
- Silver staining
- High dense precipitation in vestibular center (B) at 7 d after double blast exposures

5 Effect of blast exposure on auditory cortex



- Immunohistochemistry on brain sections
- at 6 h and 7 d after TBI
- Expression of GFAP (green) and CCR2 (red)
- Scale bar = 200 μ m

6 Blast-induced inflammation related gene expression



- Tissues were collected at 6 h after double blast exposures
- Groups: sham and 2BOPs, 3 rats for each group

CONCLUSIONS

- Peripheral and central auditory and vestibular systems are vulnerable to a blast-related injury
- Compared to sham controls and single blast exposure, repeated blasts caused significant motor coordination deficits and damage to middle and inner ear
- Severities of middle and inner ear injuries were blast intensity-dependent
- Inner ear hemorrhage was not observed at 6 h post exposure (data not showed), but was presented significantly at 24 h and up to 14 days that indicated an occurring of the secondary injury
- Repeated blast exposures elicit inner ear and CNS inflammation

DISCLAIMER There is no objection to its presentation. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals.